

CANCER RESEARCH

VOLUME 8

NUMBER 7

JULY, 1948

A MONTHLY JOURNAL

REPORTING CANCER RESEARCH

CONTENTS

HERBERT SILVERSTONE. The Levels of Carcinogenic Azo Dyes in the Livers of Rats Fed Various Diets Containing *p*-Dimethylaminoazobenzene. Relationship to the Formation of Hepatomas 301

HERBERT SILVERSTONE. The Effect of Rice Diets on the Formation of Induced and Spontaneous Hepatomas in Mice 309

HUGH J. CREECH, Ph.D., MARY ALICE HAMILTON, Ph.D., and IRENE COREY DILLER, Ph.D. Comparative Studies of the Immunological, Toxic and Tumor-Necrotizing Properties of Polysaccharides from *Serratia marcescens* (*Bacillus prodigiosus*) 318

HUGH J. CREECH, Ph.D., MARY ALICE HAMILTON, Ph.D., EDWIN T. NISHIMURA, M.D., and REED F. HANKWITZ, JR., B.S. The Influence of Antibody-Containing Fractions on the Lethal and Tumor-Necrotizing Actions of Polysaccharides from *Serratia marcescens* (*Bacillus prodigiosus*) 330

S. C. PAN, M.B. and W. U. GARDNER, Ph.D. Carcinomas of Uterine Cervix and Vagina in Estrogen- and Androgen-Treated Hybrid Mice 337

JOSEPH M. BUTROS, M.A. The Effect of Methylcholanthrene on the Viscosity of Paramecium 347

THE OFFICIAL ORGAN OF THE
AMERICAN ASSOCIATION FOR CANCER RESEARCH, INC.

CANCER RESEARCH

This journal is sponsored by The American Association for Cancer Research, Inc.; The Anna Fuller Fund; Cancer Research Division, Donner Foundation, Incorporated; The Jane Coffin Childs Memorial Fund for Medical Research; and The Elsa U. Pardee Foundation.

Editors

BALDUIN LUCKÉ, *Editor*

MORTON McCUTCHEON, *Associate Editor*

Editorial Committee

J. J. BITTNER, *University of Minnesota Medical School*
A. BRUNSCHWIG, *Memorial Hospital, New York City*
E. V. COWDRY, *Barnard Free Skin and Cancer Hospital, St. Louis*
D. R. COMAN, *University of Pennsylvania School of Medicine*
R. P. CUSTER, *Presbyterian Hospital, Philadelphia*
L. I. DUBLIN, *Metropolitan Life Insurance Company, New York City*
F. DURAN-REYNALS, *Yale University School of Medicine*
G. FAILLA, *College of Physicians and Surgeons, New York City*
L. F. FIESER, *Harvard University*
A. JAMES FRENCH, *University of Michigan, Ann Arbor*
J. FURTH, *Veterans Administration Hospital, Dallas, Texas*
W. U. GARDNER, *Yale University School of Medicine*

H. S. N. GREENE, *Yale University School of Medicine*
J. P. GREENSTEIN, *National Cancer Institute*
J. L. HARTWELL, *National Cancer Institute*
F. L. HAVEN, *Strong Memorial Hospital*
J. G. KIDD, *Cornell University Medical College, New York City*
E. C. MACDOWELL, *Carnegie Institution of Washington, Cold Spring Harbor, L.I.*
G. B. MIDER, *Strong Memorial Hospital*
E. G. MILLER, JR., *College of Physicians and Surgeons, New York City*
J. J. MORTON, JR., *Strong Memorial Hospital*
E. H. QUIMBY, *College of Physicians and Surgeons, New York City*
E. W. SHRIGLEY, *Yale University*
H. L. STEWART, *National Cancer Institute*
G. H. TWOMBLY, *Memorial Hospital, New York City*
S. WARREN, *Harvard Medical School*

Published by American Association for Cancer Research, Inc.
Publication Office, 317 Maynard Street, Ann Arbor, Michigan

The annual subscription rates for one volume are: To members of the American Association for Cancer Research, Inc., \$5.00; to others and to libraries, institutions, and organizations, domestic, \$7.00; Canadian, \$7.50; foreign, \$8.00. Single copies, \$1.00. Business communications, remittances, and subscriptions should be addressed to Dr. Theodore P. Eberhard, Business Manager, Jefferson Hospital, 10th and Sansom Streets, Philadelphia 7, Pa.

No responsibility is accepted by the Committee, by the Board, or by the Publishers of *Cancer Research* for opinions expressed by contributors.

Entered as second class matter December 16, 1946, at the Post Office at New Haven, Conn., under the Act of March 3, 1879. Application pending transfer to Ann Arbor, Mich.

Copyright, 1947, by Cancer Research, Inc.

CANCER RESEARCH

A MONTHLY JOURNAL OF ARTICLES AND ABSTRACTS REPORTING CANCER RESEARCH

VOLUME 8

JULY, 1948

NUMBER 7

The Levels of Carcinogenic Azo Dyes in the Livers of Rats Fed Various Diets Containing *p*-Dimethylaminoazobenzene

Relationship to the Formation of Hepatomas*

Herbert Silverstone

(From the Department of Biochemistry, University of Chicago, Chicago 37, Ill., and the Department of Cancer Research, Michael Reese Hospital, Chicago 16, Illinois)

(Received for publication December 17, 1947)

Variations in the composition of the diet influence the rate of appearance of liver tumors in rats fed certain azo dyes. The effective dietary factors and possible mechanisms involved have been reviewed by Rusch, Baumann, Miller, and Kline (14); as yet no generally accepted explanation has been advanced either for the mode of carcinogenic action or for the means by which diet affects it.

It has been shown that the rate of appearance of tumors of the skin or subcutaneous tissues, induced by carcinogenic hydrocarbons, is directly dependent on the dosage of carcinogen applied. Furthermore, there is evidence that the same relationship may be valid for the induction of hepatomas by means of azo dyes. It may be that variations in the composition of the diet increase or decrease the rate of appearance of hepatomas by increasing or decreasing the level of azo dyes in the liver.

Since the azo dyes are fat soluble, one way in which diets might produce differences in dye levels in the liver is through their effect upon the lipid content of the liver. Any diet tending to increase liver lipids might lead to high concentration of azo dyes in the liver, and in this way augment the formation of hepatomas. Consideration of the various dietary conditions known to affect the formation of induced hepatomas suggests that at least some of the diets augmenting tumor formation would tend

to increase liver lipids. Indeed, Gyorgy, Poling, and Goldblatt (2) concluded that the action of casein, in protecting against pathological changes induced by *p*-dimethylaminoazobenzene, may be due to its lipotropic activity.

The present experiments were designed to examine the relationship between hepatoma formation and the levels of carcinogenic azo dye and liver lipids in rats fed *p*-dimethylaminoazobenzene. Diets known to effect different rates of formation of hepatomas were utilized. Each diet was fed to two groups of rats; one group was employed to determine the influence of the diet on tumor incidence, the other the effect of the diet on the level of carcinogenic azo dye and lipids in the liver.

PROCEDURES

The diets are shown in Table I. Diets g 71 and g 72, brown rice diets, differed in that the latter contained 15 per cent brewer's yeast. Diets g 73, g 74, and g 75 differed in the levels of protein (casein), fat (partially hydrogenated oils) and carbohydrate (cornstarch), but were designed to supply equal amounts of salts and vitamins relative to equal caloric intakes. Diets g 73 and g 74, contained equal amounts of fat but differed in protein content and, reciprocally, in carbohydrate. Diets g 74 and g 75 contained equal amounts of protein with respect to equal caloric intakes but differed in the amounts of fat.

Diets g 71 and 72 were utilized because of the known effect of supplemental yeast in retarding the formation of liver tumors induced in rats fed *p*-dimethylaminoazobenzene in a rice diet (17). Similarly, available data (5, 12) indicated that the rate

*Submitted to the Division of Biological Science of the University of Chicago, in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

Presented at the 38th Annual Meeting of the American Association for Cancer Research, Chicago, Ill., May 16 and 17, 1947.

TABLE I: COMPOSITION OF DAILY RATIONS

	Brown rice diets		Grams of food per rat per day	Partially purified diets		
	g71	g72		g73	g74	g75
Ground brown rice	15.68	13.28	Casein	3.6	1.2	1.2
Brewer's yeast		2.4	Cornstarch	4.2	6.6	13.3
Olive oil	.24	.24	Kremax	2.76	2.76	0
Cod liver oil	.08	.08	Brewer's yeast	.48	.48	.48
			Salts	.48	.48	.48
			Ruffex	.24	.24	.24
			Olive oil	.18	.18	.24
			Cod liver oil	.06	.06	.06
Total	16.0	16.0		12.0	12.0	16.0
			Calculated data*			
Protein—%	8	14		30	10	8
Fat—%	4	4		25	25	2
Riboflavin μgm. per gm.	0.5	7.0		2.4	2.2	1.6
Calories	58	58		58	58	58

* Brewer's yeast—Anheuser Busch's Strain K Yeast; Casein—Borden's Labco Vitamin Free; Salts—Wesson (18); Kremax—Armour's partially hydrogenated cottonseed-soybean oil. The caloric values, the protein and fat, and the riboflavin of the diet were computed from the manufacturers' analysis and from "Tables of Food Composition," U.S. Department of Agriculture, 1945, Misc. Publication No. 572.

of appearance of hepatomas in rats fed diet g 73 (high protein) should be retarded with respect to that in rats fed diet g 74 (low protein). Also, it was expected that g 74 (high fat) would increase the rate of hepatoma formation over that in rats fed g 75 (low fat) (6, 13).

p-Dimethylaminoazobenzene dissolved in warmed olive oil was incorporated in the diets at a level of 0.06 per cent of the weight of the diet. The diets were prepared by mixing the weighed ingredients with sufficient water to make easily molded mashers which were spread in pans, cut into blocks of appropriate size and stored in a refrigerator at 36 to 40° F. The diets were prepared once each week and fed daily. Food consumption was determined by weighing back, at 3 day intervals, the food remaining in the cages.

The rats employed were 11 to 12 week old Sprague Dawley males. They were housed two in a cage and had water available at all times. Each of the diets was fed to two groups of rats; groups of 24 rats were employed in a series in which hepatoma formation was studied, and groups of 5 rats in a series in which lipid and azo dye levels in the liver were determined.

Each of the diets containing 0.06 per cent *p*-dimethylaminoazobenzene was fed to 24 rats for 4 months; the dye was then removed from the diets and the rats maintained on the dye-free rations either until they died (with hepatomas) or were sacrificed because of weakness associated with palpable hepatic masses, or until the end of the experiment at 6 months. The animals were weighed and inspected at one week intervals. All animals were inspected at autopsy for hepatomas; these were recognized grossly and confirmed by microscopic examination.

The same rations containing *p*-dimethylaminoazobenzene were also fed to groups of 5 rats for 8 weeks. During the last week, the rats were sacrificed and the livers of the individual animals analyzed for total lipids, cholesterol, lipid phosphorus, and azo dyes. The rats were not deprived of food before being sacrificed. It was assumed that by the eighth week, the livers would have attained a relatively representative or average state with respect to metabolism of liver lipids and the azo dyes, and, on the basis of available reports, would not yet have developed any striking structural changes or neoplasia.

Liver lipids.—The animal to be analyzed was lightly anesthetized with ether and then decapitated; this resulted in fairly effective exsanguination of the hepatic tissue. Pieces of liver weighing 3 to 4 gm. were cut from the several lobes (to insure adequate sampling), weighed to the nearest centigram, then ground with sand and 10 cc. of hot acetone in a mortar. This was transferred quantitatively with the aid of 10 cc. of acetone to a 100 cc. flask where it was further extracted with two successive portions of ethyl ether and once with petroleum ether (Skellysolve F). The successive extracts were filtered through a cotton-plugged funnel into a second 100 cc. flask. The flask containing the combined extracts was covered with a watch glass and placed in a water bath at 65 to 75° C. until the odor of acetone was no longer detectable. One drop of 50 per cent KOH was added to the residue which was then extracted 3 times with 5 to 10 cc. portions of petroleum ether. The combined petroleum ether extracts were collected in a weighed 50 cc. flask and all but the last few milliliters evaporated off on a water bath. The extract was dried to constant weight in a vacuum desiccator.

cator over P_2O_5 , under CO_2 at 40 to 50° C.; the flask was reweighed to determine the total lipids. As soon as possible (generally within 5 minutes after removal from the vacuum desiccator), the lipids were redissolved in petroleum ether. Aliquots of this solution were employed to determine lipid phosphorus by the method of Fiske and Subbarow (1), and cholesterol by the method of Schoenheimer and Sperry (15).

The average deviation of duplicate samples was 5 per cent. With respect to recovery, the overall efficiency was 92 per cent to 96 per cent compared with lengthier and more vigorous procedures such as extraction in a soxhlet with ethanol, chloroform and ether, or ether extraction of the alkali-digested tissue.

Azo dyes.—The analysis of the livers for azo dye was based on the following considerations: Miller and his associates (11) have shown that *p*-dimethylaminoazobenzene (DAB) is demethylated *in vivo* to *p*-monomethylaminoazobenzene (MAB) and *p*-aminoazobenzene (AB). This demethylation also occurs *in vitro* in the diets themselves (4). DAB and MAB are equally carcinogenic when fed to rats while AB is a relatively feeble carcinogen (8, 16). The analytical method employed in the present experiment determined the total azo dye (DAB, MAB, and AB), and AB alone; the difference between these 2 determinations represents the sum of DAB and MAB, that is, the total carcinogenic azo dye.

DAB, MAB, and AB may be quantitatively extracted from petroleum ether solutions by strong HCl in ethanol or water, forming red to orange-red solutions. The absorption of light by such solutions (containing less than 3 μ gm. of the dyes per cc.) follows Beer's Law, both with respect to changing concentrations and to the additivity of the optical densities of the individual dyes, in the light range from 500 to 520 $m\mu$. In equal concentrations DAB and MAB have very nearly equal optical densities (7). AB diazotizes and couples with α -naphthol to form a purple colored *bis*-azo dye which in alkaline alcohol solutions has maximal light absorption at 580 $m\mu$; at this wave length the light absorption follows Beer's Law. By this reaction AB can be determined without interference by DAB or MAB. These two procedures afford a means of determining the amount of DAB and MAB, the carcinogenic azo dyes, in a solution containing all 3 dyes.

By successive extraction with acetone and ether, the azo dyes added to liver are quantitatively extracted along with the hepatic lipids. The solvents

can be removed from this extract and the azo dyes and lipids dissolved in petroleum ether. The azo dyes in this petroleum ether solution cannot be directly determined by HCl extraction or diazotization and coupling because of marked interference by phospholipids: As little as 2 mgm. of phospholipid per cc. of solution reduces the amount of DAB or AB extracted by HCl by more than 50 per cent; a smaller but still considerable interference is observed in the diazotization and coupling reaction for AB. Other petroleum ether soluble substances present in animal tissues did not interfere.

The azo dyes can be quantitatively separated from the lipids (including the interfering phospholipids) by chromatography. When a petroleum ether solution of the azo dyes and liver lipids is filtered through a short column of an acid earth adsorbent, the azo dyes are adsorbed as an orange-red to red crust at the top of the column whereas only small, noninterfering amounts of the lipids remain on the column. The dyes are readily eluted from the column by means of a mixture of ethanol and petroleum ether; the eluate can then be quantitatively analyzed. Extracts of materials low in phospholipid, such as urine or inguinal depot fat, can be analyzed for the azo dyes without use of an adsorption procedure.

The details of the analytical methods employed in the present study were as follows:

Reagents:

1. Petroleum ether. Skellysolve F. (b.p., 30–60° C.)
2. 1:1 Petroleum ether-ethanol. Mixture of equal parts 95 per cent ethanol and petroleum ether.
3. Concentrated HCl
4. 1N HCl
5. 0.1% $NaNO_2$ in water, freshly prepared.
6. 0.05% — Naphthol in 1N NaOH, freshly prepared.
7. Lloyd's Reagent, a fine mesh aluminum silicate, employed as the adsorbant.

Apparatus:

1. Colorimeter. Cenco-Sheard spectrophotometer, employing test tubes (11.2 mm. i. d.) as cuvettes.
2. Calibrated centrifuge tubes, 15 ml. size.
3. Adsorption columns. The columns of Lloyd's Reagent were prepared in funnels of the type used with Gooch crucibles; the funnels have a bowl capacity of 30 ml. and stems 6 mm. by 10 cm.; the tip of the stem is constricted to a 2 mm. opening. Columns 8 to 13 mm. high were prepared in the stem by plugging the tip with cotton and tamping down the

dry adsorbant while applying suction. The funnels are held in filter flasks attached to a suction manifold.

Procedure.—4 to 5 grams of liver, cut from the several lobes and weighed to the nearest centigram, was subjected to the treatment described for determination of lipids through the stage of redissolving the dried lipid extract in petroleum ether. The column of Lloyd's Reagent was wetted with petroleum ether and the lipid extract filtered at the rate of 1 to 2 cc. per minute; the column was then washed twice with 10 cc. portions of petroleum ether. While still damp, the column was transferred to a 15 cc. centrifuge tube where it was eluted successively with 5 and 4 cc. portions of 1:1 petroleum ether-ethanol. The eluates were collected in a 15 cc. calibrated centrifuge tube.

The combined eluate was diluted to 9 cc. with 1:1 petroleum ether-ethanol; 3 cc. was transferred to a second calibrated centrifuge tube for determination of AB, while the remaining 6 cc. was used to determine the total azo dye (DAB, MAB, and AB).

The 3 cc. aliquot was diluted to 4 cc. with 1:1 petroleum ether-ethanol; 0.3 cc. of 1*N* HCl and 0.1 cc. of 0.1 per cent NaNO₂ were added and the tube shaken well. After the phases separated, sufficient ethanol was added to make the alcohol phase 2.4 cc. After 3 to 4 minutes, 0.6 cc. of the solution of α -Naphthol in 1*N* NaOH was added, the tube shaken again and the phases separated by centrifugation; the petroleum ether phase was discarded. To determine the amount of AB present in the aliquot, the absorption of light by the alkaline alcohol phase was determined at 580 m μ using a reagent blank. Although the solutions darken in air, the light absorption relative to that of the reagent blank remains constant for at least 24 hours.

To the 6 cc. aliquot, 2 cc. of concentrated HCl was added, the tube shaken vigorously and the phases separated by centrifugation; sufficient ethanol was added to make the acid phase 5 cc. in volume, following which the shaking and centrifugation were repeated; the petroleum ether phase was discarded. To determine the amount of DAB, MAB, and AB present in the aliquot, the absorption of light by the acid-alcohol phase was determined at 510 m μ .

The amount of carcinogenic azo dye (DAB plus MAB) was computed on the basis of the predetermined colorimeter constants of the 3 azo dyes in HCl solution and the colorimeter constant of the diazotized and coupled AB. The optical density of

the 6 cc. aliquot is the sum of the optical densities of the DAB, MAB, and AB present. The optical density of the AB alone was calculated from its specific measurement in the 3 cc. aliquot. The difference, considering the volumes of the aliquots, is due to the carcinogenic dyes.

Precautions.—1. The exposure to air of the dried lipid extract containing the azo dyes must be kept to a minimum, since under such conditions the dyes are destroyed by autoxidative changes of the lipids. 2. The petroleum ether extract which is filtered through the column must be free of water; even traces of water cause the Lloyd's Reagent to swell, slowing the filtration or stopping it altogether. 3. While no experiments were performed on the effect of temperature on the diazotization and coupling reaction, it was noted that when the room temperature exceeded 26° C. color development was erratic; therefore, when the temperature was above 25° C. the solutions were cooled to 20 to 25° C. in a water bath.

Limitations of the method.—With amounts of DAB and AB ranging from 4 to 10 μ gm. in the presence of one another and separately, added to 4 gm. of liver, the recovery of added dye varied from 80 to 100 per cent; average recovery was 90 per cent. By adding the dyes at successive steps in the procedure, the loss was found to be due to a summation of smaller losses. The average deviation of a series of 15 duplicate determinations, for DAB plus MAB in the livers of rats which had been fed DAB, was 7 per cent; with smaller amounts than 1 μ gm. per sample (4 duplicates) the deviations ranged from 2 to 23 per cent; between 1 and 5 μ gm. per sample (11 duplicates) the deviations ranged from 0 to 10 per cent. The tissue blank—*i.e.* apparent azo dye in the livers of animals that had not been exposed to *p*-dimethylaminoazobenzene—was less than 0.07 μ gm. per gm. of tissue. In presenting the data no correction was made for average losses due to the method or for tissue blank.

The method of extraction is not applicable to analysis for AB in the blood of animals which have been injected with or fed *p*-dimethylaminoazobenzene. When AB is added to samples of blood, plasma, or liver it is recovered in near quantitative amounts; on the other hand, the extraction procedure described here does not appreciably extract AB from the tissues of animals which have been ingesting DAB. Less than 0.2 μ gm. of AB per cc. was extracted from the blood of rats in contrast to 7 to 35 μ gm. of AB per cc. extracted from the

blood of the same animals when the extraction procedure described by Miller and Baumann (7) was used.

These authors report AB at levels of 0.07 to 2.0 μ gm. per gm. of liver in rats ingesting 0.06 per cent DAB, while our method never detected more than 0.3 μ gm. per gm. of liver in comparable animals. Whether this striking difference is due to the relative efficiencies of the methods in extracting AB from the liver itself or from the residual hepatic blood is not known. If it is due to the latter, our extraction procedure may have the advantage of eliminating residual hepatic blood as a possible interfering factor in the determination of AB in liver. At any rate, quantitative extraction of AB is not necessary for the quantitative determination of the carcinogenic azo dyes, DAB and MAB.

RESULTS

Hepatoma formation.—The effects of the diets on body growth, food intake, and hepatoma formation are shown in Table II. At 6 months (the end of the experiment) the incidence of hepatomas in the 5 groups ranged from 25 to 96 per cent. The group fed brown rice supplemented with 15 per cent yeast developed 37 per cent hepatomas in comparison with 96 per cent in the group fed brown rice alone, a statistically significant retardation.

The following differences were found among the 3 groups given the rations prepared from partially purified constituents: Of the two groups fed diets containing moderate levels of B vitamins and 25 per cent fat, the one fed 10 per cent casein developed 58 per cent hepatomas, in comparison with 25 per cent in the group fed 30 per cent casein; the difference is statistically significant. Of the two groups fed diets containing the same amounts of casein, vitamins, and salts, and equicaloric amounts of fat plus carbohydrate, the group fed 2 per cent fat developed 71 per cent hepatomas compared with 58 per cent in the group fed 25 per cent fat.

The rate of appearance and the size of the hepa-

tomas at autopsy in the several groups paralleled the final incidences of hepatomas. Before the experiment was terminated 16 of the animals in g 71 had died from or were sacrificed because of large, usually multiple, hepatic tumors, while no more than 6 rats had died or been sacrificed for the same reason in any of the other groups. While all of the hepatomas observed in g 71 and g 75 were large (1 to 5 cm.), several of the rats in the other groups had hepatomas only 5 mm. in diameter. It may be emphasized that there were no deaths from causes other than hepatomas.

The average daily food intake (and correspondingly the amount of *p*-dimethylaminoazobenzene) and body growth differed among the groups. Hepatoma incidences in groups g 71 and g 72 or in g 73 and g 74 were not related to the weight of food and dye ingested; on the other hand, the increase in tumor incidence from 58 per cent in g 74 to 71 per cent in g 75 paralleled the greater (by about 10 per cent) food and dye consumption of g 75.

There was an inverse relationship between the average change in body weight and the incidence of hepatomas. The 2 groups, g 71 and 75, developing the higher incidences of hepatomas lost weight while the other 3 groups, g 72, 73, and 74, either maintained their weight or grew. The differences in body weight were apparent as early as the fourth week of the experiment.

The differences in food intake and body growth of the several groups were due principally to the *p*-dimethylaminoazobenzene in the rations rather than to the rations themselves. This was determined by feeding the same diets, *but without the dye*, to groups of comparable rats for 4 weeks. These rats consumed all their daily rations (58 calories) and the average weight gains for the groups were from 19 to 40 gm. In contrast, the groups fed the dye-containing rations consumed from 47 to 55 calories daily with average weight changes of -34 to +19 gm. for the same period.

Liver lipids and carcinogenic azo dye.—The liv-

TABLE II: HEPATOMA FORMATION, BODY GROWTH, AND FOOD INTAKE OF RATS INGESTING 0.06% *p*-DIMETHYLAMINO-AZOBENZENE IN VARIOUS DIETS. 24 RATS IN EACH GROUP

Group	Mean initial weight, gm.	Weight change (gm.) with <i>p</i> -dimethylaminoazobenzene in diet		Mean daily food intake		Rats with hepatomas by 6th month	
		At 4 weeks	At 4 months	Gm.	Calories	Number	Percent
g71—Brown rice	280	-10 to -56 (-34)	-70 to -6 (-27)	13	47	23	96
g72—Brown rice, yeast	289	-34 to +24 (+4)	+6 to +66 (+40)	15	54	9	37
g73—High protein, high fat	288	-18 to +42 (+19)	+12 to +84 (+49)	11.5	55	6	25
g74—Low protein, high fat	285	-42 to +22 (-4)	-20 to +75 (+6)	11.5	55	14	58
g75—Low protein, low fat	277	-50 to +6 (-24)	-58 to +14 (-24)	13	47	17	71

ers of the animals sacrificed during the eighth week for analytical purposes were grossly normal in appearance; in some of the animals fed the brown rice diet, g 71, and the low protein synthetic rations, g 74 and g 75, the livers were slightly paler than normal, and the edges slightly rounded. No association was observed between these changes and any of the analytical findings.

For convenience in considering the data on liver lipids and carcinogenic azo dye, the groups are tabulated in order of increasing incidence of hepatomas. The data on liver lipids are shown in Table III. The only animals revealing increased levels of total lipids were those fed the high fat-low protein ration, g 74. None of the other rations led to any noteworthy increase in hepatic lipids under the conditions of the experiment. Comparable rats fed the same rations for the same period of time, *but without p-dimethylaminoazobenzene*, revealed, in general, slightly lower levels of total liver lipids, about 9.5 per cent in those fed the g 74 ration and 3.5 per cent in those fed the other rations.

Comparison of the total liver lipids of the several groups with the incidences of hepatomas, suggests that increased total liver lipids are not essential to increased development of hepatomas. Thus, the retarding effect on the rate of formation of *p*-dimethylaminoazobenzene-induced hepatomas, caused by supplementing a brown rice diet with 15 per cent yeast is not dependent on differences in total lipids of the liver. Furthermore, the rats fed g 74, the only ration that produced an increase in total lipids, developed an intermediate percentage of hepatomas. As with total lipids, the liver cholesterol values do not show any association with the formation of liver tumors.

In these experiments the average values for lipid phosphorus appear to be inversely related to the incidence of tumors. The diets resulting in the lowest lipid phosphorus (g 71 and g 75) led to a greater hepatoma development; the ration effect-

ing the lowest incidence of hepatomas (g 73) resulted in an average lipid phosphorus level equivalent to that found in the livers of rats fed a presumably complete ration (Purina fox chow checkers) without *p*-dimethylaminoazobenzene.

The levels of carcinogenic azo dye (DAB plus MAB) found in the livers of the 25 individual rats fed the 5 experimental diets are shown in Table IV. The diets resulting in higher average levels of carcinogenic dye in the liver also effected higher incidences of hepatomas: the average values of 0.32, 0.22, 0.46, 0.51, and 0.89 μ gm. of carcinogenic dye per gram of liver correspond to percentage incidences of hepatomas of 25, 37, 58, 71, and 96, respectively. The group mean levels of carcinogenic azo dye differed significantly among themselves ($F = 3.66$, P less than 5 per cent), although there were only 5 rats in each of the groups analyzed for carcinogenic azo dye in the liver, and the individual values overlapped. Thus, in these experiments, there appears to be a direct relationship between the average levels of carcinogenic dye in the liver and the development of hepatomas in rats fed various diets containing *p*-dimethylaminoazobenzene.

DISCUSSION

The direct relationship between the level of carcinogenic azo dye in the liver and the rate of hepatoma formation observed in these experiments should be discussed in connection with the data reported by Miller and his associates (9). They have shown that hydrogenated coconut oil, mineral oil, detergents, or high riboflavin in the diet tend to protect rats against the formation of hepatomas induced by *p*-dimethylaminoazobenzene. They also examined the effects of these substances on the levels of DAB, MAB, and AB in the livers of rats and concluded that "although the average levels of the dyes in liver and blood were somewhat lower in animals that received protective factors the vari-

TABLE III: LIVER LIPIDS IN RATS FED DIETS CONTAINING 0.06% *p*-DIMETHYLAMINOAZOBENZENE FOR 8 WEEKS; 5 RATS IN EACH GROUP

Diet	Mean* weight of rats	Mean liver weight per 100 gm. rat, gm.	Total lipids, %	Liver lipids based on wet weight		Lipid phosphorus mgm. %
				Free Cholesterol %	Total	
g73—High protein, high fat	326	3.8	3.8–4.8 (4.5)	.19–.28 (.23)	.26–.31 (.28)	85–119 (106)
g72—Brown rice, yeast	310	3.7	3.8–5.3 (4.6)	.15–.22 (.19)	.20–.34 (.28)	83–99 (96.5)
g74—Low protein, high fat	288	3.6	7.3–14.4 (10.7)	.16–.24 (.21)	.33–.54 (.45)	75–103 (93.0)
g75—Low protein, low fat	261	3.3	4.2–5.8 (4.8)	.19–.22 (.21)	.30–.45 (.35)	78–87 (82.5)
g71—Brown rice	270	3.4	3.2–5.7 (4.4)	.16–.23 (.20)	.22–.42 (.30)	67–94 (84.6)

* The rats of each group averaged 260 to 270 gm. at the start of the experiment.

TABLE IV: "CARCINOGENIC DYE" (DAB PLUS MAB) IN LIVERS OF RATS INGESTING DIETS CONTAINING 0.06% *p*-DIMETHYLAMINOAZOBENZENE FOR 8 WEEKS

Diet	Incidence of hepatomas (Table II)	Micrograms of carcinogenic dye per gram of liver	
		Individual rats	Average
g73—High protein, high fat	25%	0.05, 0.14, 0.22, 0.39, 0.80	0.32
g72—Brown rice, yeast	37%	0.06, 0.16, 0.21, 0.31, 0.33	0.22
g74—Low protein, high fat	58%	0.06, 0.11, 0.32, 0.70, 1.11	0.46
g75—Low protein, low fat	71%	0.12, 0.42, 0.54, 0.68, 0.80	0.51
g71—Brown rice	96%	0.46, 0.67, 1.02, 1.03, 1.25	0.89

ability and overlapping of figures make it difficult to draw any final conclusion".

In our experiments, the feeding of 5 different diets, each containing 0.06 per cent *p*-dimethylaminoazobenzene, resulted in average hepatic carcinogenic dye levels that differed significantly among themselves. The range of average values—from 0.22 to 0.89 μ gm. of carcinogenic azo dye per gram of liver—was obtained with a small number of animals, and the individual values overlapped. Nevertheless, if tumor formation in the liver is dependent on the dosage of carcinogen that comes in contact with that organ, it is probable that the wide range of average dye values observed in our experiments was sufficient to effect different rates of hepatoma formation. Actually a relatively direct relationship was observed: the average values of 0.32, 0.22, 0.46, 0.51, and 0.89 μ gm. of carcinogenic dyes in the liver corresponded to percentage incidences of hepatomas of 25, 37, 58, 71, and 96, respectively.

Although there are a few studies on the dependence of the rate of formation of liver tumors on the dosage of the injected or ingested carcinogen, there is only one (9) in which the hepatic concentration of the dye was also studied. It was found that feeding 2 pairs of rats diets containing 0.06 per cent and 0.03 per cent *p*-dimethylaminoazobenzene, resulted in average hepatic dye levels (DAB plus MAB) of 0.33 and 0.18 μ gm. respectively—a direct and almost proportional relationship (8). These concentrations of dye in the diet—0.06 per cent and 0.03 per cent—produced 80 and 0 per cent hepatomas, respectively (10).

Evidence points to the dependence of hepatoma formation on the dosage of carcinogen administered. However, if such dependence is to be related to the concentrations of carcinogen in the liver, more basic data on the relationship of dye administered, hepatic dye levels, and rate of hepatoma formation in graded dosage experiments are needed, as suggested by Miller and associates (9). In addition, this should be coupled with further and more extensive work on the effects of varying the diet (all containing the same concentration or amount of carcinogen) on the concentration of carcinogenic dyes in the liver, and subsequent hepatoma formation.

In view of our experiments and those of Miller and co-workers, it appears that in some instances the composition of the diet may affect the rate of appearance of hepatomas through its effect upon the concentration of carcinogenic azo dye in the liver. However, there is no reason to assume that all dietary modifications produce their effects through this means.

The present experiments offer no support to the assumption that total hepatic lipids are a significant factor in the mechanism whereby diet influences the rate of appearance of hepatomas induced by *p*-dimethylaminoazobenzene: Neither the level of carcinogenic azo dye nor the incidences of hepatomas at 6 months were related to the level of total lipids or cholesterol in the liver. There was an inverse relationship between average liver lipid phosphorus and incidence of hepatomas—livers of rats fed diets which induced higher incidences of hepatomas had significantly depressed concentrations of lipid phosphorus.

In confirmation of the results of other investigations, replacing 15 per cent of a brown rice diet with yeast retarded the appearance of hepatomas. Furthermore, the formation of hepatomas was significantly inhibited by increasing the casein content of a partially purified ration from 10 per cent to 30 per cent. Whether increased dietary casein protects against induction of hepatomas by *p*-dimethylaminoazobenzene depends upon several dietary factors such as the levels of casein being compared and the level of B-vitamins, particularly riboflavin (3, 14).

Group g 75 fed the low fat diet developed about the same incidence of hepatomas as group g 74 fed the high fat ration. This is not in agreement with previous conclusions that high dietary fat accelerates hepatoma formation (6, 13). However, the nature of the fat utilized determines the effect; increasing the level of corn oil from 5 to 20 per cent accelerated the appearance of hepatomas, but replacing 5 per cent corn oil with 20 per cent Crisco or lard did not effect any increase in the incidence of hepatomas (6). Our data may be related to these observations since Kremax, a fat similar to Crisco, was used to increase the fat content of the diets.

SUMMARY

The present investigation was designed to study the possibility that diets affect the formation of hepatomas induced in rats by *p*-dimethylaminoazobenzene through modifying the level of carcinogenic azo dye in the liver. The possibility that the liver lipid concentration is a significant factor in the dietary modification of carcinogenicity was also considered. A method for the determination of carcinogenic azo dye (*p*-dimethylaminoazobenzene plus *p*-monomethylaminoazobenzene) in the livers of rats fed *p*-dimethylaminoazobenzene is presented.

Using five diets that resulted in incidences of liver tumors ranging from 25 to 96 per cent at 6 months, it was found that neither the percentage of hepatomas nor the level of carcinogenic azo dye in the liver were associated with the level of total lipids or cholesterol of the liver. Hepatic lipid phosphorus was inversely related to the incidence of hepatomas.

In rats fed the five different diets, average levels of carcinogenic azo dye per gram of liver—0.32, 0.22, 0.46, 0.51, and 0.89 $\mu\text{gm.}$ —in animals sacrificed at 8 weeks, corresponded to the percentage incidences of hepatomas at 6 months of 25, 37, 58, 71, and 96, respectively. Thus, in these experiments, there appeared to be a direct relationship between the level of carcinogenic dye in the liver (tissue dosage of carcinogen) and the rate of formation of liver tumors.

ACKNOWLEDGMENT

This work was done under the direction of Dr. T. F. Gallagher, Department of Biochemistry, University of Chicago, and Dr. Albert Tannenbaum, Department of Cancer Research, Michael Reese Hospital, Chicago, Illinois.

REFERENCES

1. FISKE, C. H., and SUBBAROW, Y. The Colorimetric Determination of Phosphorus. *J. Biol. Chem.*, **66**: 375-400. 1925.
2. GYORGY, P., POLING, E. C., and GOLDBLATT, H. Necrosis, Cirrhosis and Cancer of Liver in Rats Fed a Diet Containing Dimethylaminoazobenzene. *Proc. Soc. Exper. Biol. & Med.*, **47**:41-44. 1941.
3. HARRIS, P. N., KRAHL, M. E., and CLOWES, G. H. A. *p*-Dimethylaminoazobenzene Carcinogenesis with Purified Diets Varying in Content of Cysteine, Cystine, Liver Extract, Protein, Riboflavin and other Factors. *Cancer Research*, **7**:162-175. 1947.
4. KENSLE, C. J., MAGILL, J. W., and SUGIURA, K. The Metabolism of *N,N*-Dimethyl-*p*-Aminoazobenzene and Related Compounds. *Cancer Research*, **7**:95-98. 1947.
5. KENSLE, C. J., SUGIURA, K., YOUNG, N. F., HALTER, C. R., and RHOADS, C. P. Partial Protection of Rats by Riboflavin with Casein Against Liver Cancer Caused by Dimethylaminoazobenzene. *Science*, **93**: 308-310. 1941.
6. KLINE, B. E., MILLER, J. A., RUSCH, H. P., and BAUMANN, C. A. Certain Effects of Dietary Fats on the Production of Liver Tumors in Rats Fed *p*-Dimethylaminoazobenzene. *Cancer Research*, **6**:5-7. 1946.
7. MILLER, J. A., and BAUMANN, C. A. The Determination of *p*-Dimethylaminoazobenzene, *p*-Monomethylaminoazobenzene and *p*-Aminoazobenzene in Tissue. *Cancer Research*, **5**:157-161. 1945.
8. MILLER, J. A., and BAUMANN, C. A. The Carcinogenicity of Certain Azo Dyes Related to *p*-Dimethylaminoazobenzene. *Cancer Research*, **5**:227-234. 1945.
9. MILLER, J. A., KLINE, B. E., and RUSCH, H. P. The Inhibition of the Carcinogenicity of *p*-Dimethylaminoazobenzene by Certain Detergents and the Effect of Diet on the Levels of Azo Dyes in Rat Tissues. *Cancer Research*, **6**:674-678. 1946.
10. MILLER, J. A., KLINE, B. E., RUSCH, H. P., and BAUMANN, C. A. The Effect of Certain Lipids on the Carcinogenicity of *p*-Dimethylaminoazobenzene. *Cancer Research*, **4**:756-761. 1944.
11. MILLER, J. A., MILLER, E. C., and BAUMANN, C. A. On the Methylation and Demethylation of Certain Carcinogenic Azo Dyes in the Rat. *Cancer Research*, **5**:162-168. 1945.
12. MILLER, J. A., MINER, D. L., RUSCH, H. P., and BAUMANN, C. A. Diet and Hepatic Tumor Formation. *Cancer Research*, **1**:699-708. 1941.
13. OPTIE, E. L. The Influence of Diet on the Production of Tumors of the Liver by Butter Yellow. *J. Exper. Med.*, **80**:219-230. 1944.
14. RUSCH, H. P., BAUMANN, C. A., MILLER, J. A., and KLINE, B. E. Experimental Liver Tumors. A.A.A.S. Research Conference on Cancer. Washington, D.C.: American Association for the Advancement of Science, 1945. p. 267-290.
15. SCHOENHEIMER, R., and SPERRY, W. M. A Micro-method for the Determination of Free and Combined Cholesterol. *J. Biol. Chem.* **106**:745-760. 1934.
16. SUGIURA, K., HALTER, C. R., KENSLE, C. J., and RHOADS, C. P. Observations on Rats Fed with Compounds Related to Dimethylaminoazobenzene. *Cancer Research*, **5**:235-238. 1945.
17. SUGIURA, K., and RHOADS, C. P. Experimental Liver Cancer in Rats and its Inhibition by Rice-Bran Extract, Yeast, and Yeast Extract. *Cancer Research*, **1**:3-16. 1941.
18. WESSON, L. G. A Modification of the Osborn-Mendel Salt Mixture Containing Only Inorganic Constituents. *Science*, **75**:339-340. 1932.

The Effect of Rice Diets on the Formation of Induced and Spontaneous Hepatomas in Mice*

Herbert Silverstone

(From the Department of Biochemistry, University of Chicago, Chicago 37, Ill., and the Department of Cancer Research,** Michael Reese Hospital, Chicago 16, Illinois)

(Received for publication March 9, 1948)

By 1935, various investigators (9) principally Kinosita and his co-workers had established the hepato-carcinogenic activity of *o*-aminoazotoluene and *N,N*-dimethyl-*p*-aminoazobenzene. These azo dyes, administered in the diet or by subcutaneous injection, elicit changes—including neoplasms—in the livers of rats and mice and perhaps in the livers of other species. Subsequently it was established that in the rat the formation of hepatomas induced by *o*-aminoazotoluene or *p*-dimethylaminoazobenzene is strikingly dependent on the quality of the diet. For example, dried brewer's yeast (27, 5) or liver (18, 19) added in suitable amounts to a rice diet effectively inhibit, in comparison with an unsupplemented rice diet, the formation of liver cancer in rats being fed either of these azo dyes. The investigations of Sugiura and Kensler (26), of Rusch and associates (16, 22), and of others, employing diets consisting wholly or in part of purified food stuffs such as casein, crystalline B vitamins, etc., have led to the conclusion that diets containing high levels of protein and riboflavin protect against the toxic and carcinogenic action of *p*-dimethylaminoazobenzene in rats. The protective action of dried yeast or liver is generally attributed to their high content of protein and riboflavin.

The present studies with mice were initiated to determine whether in this species also the quality of the diet influences the formation of hepatomas, either induced by an azo dye or occurring spontaneously. Four experiments were performed: Hepatomas were induced by *o*-aminoazotoluene in three of the experiments. In the fourth, the spontaneous hepatoma was studied; that is, a mouse strain in which hepatomas form without exposure to a known carcinogen was employed, and no azo dye was administered. *Db*a strain mice were used in the experiments on induced hepatomas; spon-

taneous hepatomas are not usual in these animals even by 18 months of age. *o*-Aminoazotoluene was selected as the carcinogenic azo dye since mice have been shown to be more susceptible to the hepatoma inducing action of this compound than to that of *p*-dimethylaminoazobenzene, (2, 4). In the experiment with the spontaneous hepatomas, C3H male mice were used; these mice develop an appreciable incidence of spontaneous hepatomas by 13 months of age (1).

In each of the experiments two diets were compared: One consisted principally of rice, and the other was composed of a mixture of commercial foodstuffs with Purina fox chow as a base. Although the two diets differed in many particulars, principal attention is drawn to the differences in protein and riboflavin content because of the demonstrated significance of these dietary components in the formation of hepatomas in rats fed *p*-dimethylaminoazobenzene. The diets composed with Purina fox chow contained at least twice as much protein and 5 to 10 times as much riboflavin as the rice diets.¹ From the extensive experience on the role of nutrition in the formation of liver cancer in rats fed *p*-dimethylaminoazobenzene, it was expected that hepatomas would appear more rapidly in the mice fed the rice diets.

Considerable work has been done in attempting to unearth the mechanisms involved in the dietary influence on the rate of hepatoma formation (22). It is probable that the rate of carcinogenesis in the liver, as in other tissues, is dependent, in part, on the concentration or amount of carcinogen to which the specific tissue is exposed. If so, the effects of diets on liver tumor formation may be dependent on the action of the diets in modifying the levels of the azo dye in the liver. Since the azo dyes are

* Submitted to the Division of Biological Sciences of the University of Chicago, in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

** Supported, in part, by the Michael Reese Research Foundation and the Foundation for Cancer Research.

¹ The composition of the diets, *i. e.* the content of protein, riboflavin, etc., was not determined analytically but was calculated from data supplied by the manufacturers of the foodstuffs and from data in "Tables of Food Composition". U. S. Dept. of Agriculture, 1945, Miscellaneous Publication No. 572.

fat soluble, it appeared possible that the levels of liver carcinogen, (and, consequently, hepatoma formation) might be directly dependent on the concentration of lipids in the liver. For these reasons the effects of the experimental diets on the levels of *o*-aminoazotoluene and lipids in the liver were studied. Some of these data are presented because of their general interest even though they could not be correlated with the effects of the diets on hepatoma formation.

METHODS

All mice were of inbred strains raised in our laboratory. They were housed in groups of 5 in solid bottom cages with bedding of wood shavings, and were fed Purina fox chow checkers from weaning until transfer to the experimental diets.

The experimental diets, with the exception of the Purina fox chow checkers, were prepared by mixing the weighed ingredients with sufficient water to make an easily moulded mash; this was spread in pans, cut into blocks of appropriate size, and stored in a refrigerator at 40° F. The diets were prepared once each week and fed daily. For convenience, the diets composed principally of rice are designated as the R or rice diets, and those composed of Purina fox chow with or without other foodstuffs are indicated as C or chow diets. Water was available to the mice at all times.

During the course of the experiment the animals were weighed and inspected at biweekly intervals. All animals were examined *post mortem*. Hepatomas were recognized both grossly and by microscopic sections. Other details regarding the mice, the diets, and the administration of carcinogen are given in the descriptions of the individual experiments.

At various times during the experiment, mice of the several groups were sacrificed and their livers examined for morphologic changes and analyzed for lipids and *o*-aminoazotoluene. Occasionally, brain, kidney, abdominal depot fat, urine, and blood were analyzed. In animals which were injected subcutaneously, the residue of the injected oil and *o*-aminoazotoluene was found in cysts, in various stages of organization and adhering to the underside of the skin; in several series these "injection sites" together with the adjacent skin were analyzed to determine the rate of disappearance of subcutaneously administered dye. The analytical procedures were similar to those previously reported (23). For tissues other than blood, these procedures consisted essentially of the following steps: (a) Simultaneous extraction of the lipids and azo dye

by means of hot acetone, ethyl ether, and petroleum ether, followed by gravimetric determination of the total lipids. (b) Separation of the *o*-aminoazotoluene from the lipids by chromatographic adsorption; diazotization and coupling of the isolated dye with α -naphthol, and colorimetric quantitation of the resulting *bis*-azo dye.

The procedure for extraction of the dye from the tissues failed completely when applied to blood, which was consequently extracted by a modification of the method of Miller and Baumann (14). Fifteen-tenths to 1.0 cc. of blood drawn from the vena cava into a heparinized syringe, was mixed with 2 cc. of 95 per cent ethanol and 8 cc. of 1N NaOH; this was extracted 3 or 4 times with 5 to 10 cc. volumes of petroleum ether; the *o*-aminoazotoluene present in the combined petroleum ether extracts was then determined by the colorimetric procedure indicated above. Recovery of known amounts (between 1 and 20 micrograms) of *o*-aminoazotoluene added to the various tissues ranged from about 85 per cent for liver to about 95 per cent for urine, blood, and depot fat. The sensitivity of the method was not limited by the amount of tissue analyzed but only by the amount of *o*-aminoazotoluene in the sample: Less than 0.5 microgram of the dye per analytical sample could not be determined satisfactorily.

EXPERIMENTAL

HEPATOMA FORMATION

Experiment 1.—Thirty dba male mice, 10 weeks of age, were divided into 2 equal groups. Group 1C was fed a diet consisting of 25 per cent Purina fox chow meal, 25 per cent skimmed milk powder, 3.1 per cent Kremax² (partially hydrogenated cottonseed oil), and 46.9 per cent cornstarch; this ration contained 15 per cent protein and 5 μ gm. riboflavin per gm. of food. Group 1R was fed a ration of 91 per cent white rice flour, 1.5 per cent dried brewer's yeast, 1.5 per cent cod liver oil, 3 per cent Kremax, and 3 per cent salts (30), and contained 7 per cent protein and 1 μ gm. riboflavin per gm. of food. The 1R diet was designed to simulate the brown rice plus carrot diet which, in rats fed *p*-dimethylaminoazobenzene or *o*-aminoazotoluene, promotes a high rate of hepatoma formation.

One month after institution of the diets, each mouse was injected subcutaneously in the interscapular area with 10 mgm. of *o*-aminoazotoluene³ dissolved in 0.2 cc. olive oil; 9 such injections were given at 1 month intervals. Eleven months after

²Furnished generously by Armour and Co., Chicago.

³*o*-Aminoazotoluene was obtained from Eastman Kodak Co.

the first injection of the dye, the surviving mice—13 in group 1C and 14 in group 1R—were sacrificed and examined for hepatomas. The observed incidences of hepatomas and the average weights of the mice during the course of the experiment are shown in Table I.

None of the mice fed the rice diet had visible hepatomas, while 9 (70 per cent) of the mice fed the chow ration had hepatomas ranging from 3 to 10 mm. in diameter. In this experiment, the rice diet did not augment the rate of formation of hepatomas, but actually had an inhibitory effect.

taneously in dba mice); in addition, mice were sacrificed during the course of the experiment for analytical purposes and for study of morphologic changes in the livers. The experiment was terminated 52 weeks after the first injection of *o*-aminoazotoluene. The results are shown in Table I.

As in Experiment 1, the relatively inadequate rice diet did not result in an augmented rate of formation of tumors. Although the total incidences are nearly equal, among the mice surviving to 46 weeks of age slightly fewer of the group 2R mice had hepatomas—31 per cent compared to 50 per

TABLE I: THE EFFECTS OF RICE DIETS AND CHOW DIETS ON THE FORMATION OF INDUCED AND SPONTANEOUS HEPATOMAS IN MICE

TABLE 1. THE EFFECTS OF RICE DIETS AND CHOW DIETS ON THE FORMATION OF INDUCED AND SPONTANEOUS HEPATOMAS IN MICE										
Exper. number	Hepatomas produced by	Group number and diet	Average weight of mice Months after beginning of experiment					Mice with hepatomas over total mice sacrificed in indicated interval	Final incidence of hepatomas	
			0	1	3	6	9			
1.	9 subcutaneous injections at 1 month intervals of 10 mgm. o-aminoazotoluene	1C-chow	25	28	30	34	35	11 to 12 months after 1st injection		70% 0
		1R-rice	25	30	33	34	35	9/13 (70%) 0/14 (0%)		
				Weeks after 1st injection 26—45 46—52						
2.	7 subcutaneous injections at 1 month intervals of 10 mgm. o-aminoazotoluene	2C-chow	23	25	24	27	27	1/16 (6%)	10/20 (50%)	31%
		2R-rice	22	22	22	24	23	4/21 (18%)	5/16 (31%)	24%
				Weeks after 1st feeding of dye 36—40 41—45						
3.	Feeding of 0.05% o-aminoazotoluene for 11 alternate weeks over a period of 23 wks.	3C-chow	25	23	24	25	28	5/11 (45%)	11/24 (46%)	46%
		3R-rice	26	21	23	21	23	2/4 (50%)	8/20 (40%)	42%
				14 months of age						
4.	Spontaneously occurring in male mice.	4C-chow	21	24	28	32	36	3/19 (18%)		18%
		4R-rice	21	23	28	36	37	1/20 (5%)		5%

In both groups, the mice ate approximately 4 gm. of food daily and the rice diet supported body growth and "general health" as well as the chow diet.

Experiment 2.—Two groups of dba female mice, 3 months of age were employed: Group 2C (45 mice) was continued on the Purina fox chow checkers while group 2R (55 mice) was transferred to a diet of 97.75 per cent ground brown rice, 2 per cent olive oil, and 0.25 per cent cod liver oil. The fox chow checkers contained approximately 24 per cent protein and 5 μ gm. of riboflavin per gm. of food; the brown rice ration contained only 8 per cent protein and 0.5 μ gm. riboflavin per gm. of food.

Two weeks after institution of experimental diets each mouse was injected subcutaneously in the interscapular area with 0.2 cc. of olive oil containing 10 mgm. of *o*-aminoazotoluene. Seven such injections were given at approximately 1 month intervals. During the experiment some of the mice died or were sacrificed because of palpable mammary tumors or lymphomas (which occur spon-

cent in group 2C. The difference, however, is not statistically significant and is considered suggestive only in connection with the results of Experiment 1.

The mice fed the brown rice ration did not grow as well as those fed the fox chow checkers. In addition the death rate in group 2R (rice diet) was appreciably higher than that in group 2C (chow diet); this was most noticeable during the first 26 weeks of the experiment when several of the 2R mice either died or were killed because of severe diarrhea or extensive dermatitis. The augmented death rate and depressed growth probably were due to the combination of diet and treatment with azo dye since the diet alone did not have these effects. This was determined in small groups of dba female mice fed the same diets for the same period of time but untreated with *o*-aminoazotoluene; a similar inference is indicated by the data of Experiment 4.

Experiment 3.—In the two preceding studies, the *o*-aminoazotoluene was administered by periodic injection. In the present experiment the dye was

incorporated in the diets. Each of the two groups consisted of 50 dba female mice, 14 to 16 weeks old when the experimental diets were instituted. Group 3C was fed a ration of 29 per cent Purina fox chow meal, 29 per cent skimmed milk powder, 3 per cent dried brewer's yeast, 37 per cent cornstarch, and 2 per cent olive oil; the protein content was 19 per cent and the riboflavin 10 μ gm. per gm. of food. Group 3R was fed a diet of 97.75 per cent ground brown rice, 2 per cent olive oil and 0.25 per cent cod liver oil, containing 8 per cent protein and 0.5 μ gm. of riboflavin per gm.

The animals were fed these diets without added *o*-aminoazotoluene for 4 weeks, after which the dye was added to the ration (in the olive oil) at the level of 0.05 per cent of the diet. The dye was incorporated in the diet for 1 week, omitted the following week, returned the next week, etc. This alternate incorporation and omission of the azo dye was continued for 21 weeks (dye fed during 11 alternate weeks) after which the same diets, free of *o*-aminoazotoluene, were fed until the end of the experiment 24 weeks later. This procedure of intermittent feeding was adopted because continued feeding of the dye at the concentration employed was found, in a scout experiment, to be sufficiently toxic to cause the early death of most of the animals.

The mice of group 3C (chow diet) consumed approximately 3 gm. of food daily when fed the dye-free rations, and about 2 gm. when fed the ration containing the dye. Because of considerable scattering of food, the intake of the group 3R mice (rice diet) could not be satisfactorily estimated but seemed to be less than that of the mice of group 3C.

A few of the mice fed the chow diet developed mammary or lymphatic tumors, and were sacrificed; none died of causes attributable to the toxicity of the dietary regimen. On the other hand, during the period in which the *o*-aminoazotoluene was being fed, some of the mice receiving the rice diet developed severe dermatitis and died or were sacrificed, and others died showing no gross morphologic changes. The experiment was terminated 45 weeks after the first feeding of the azo dye. The weights of the mice during the experiment and the incidences of hepatomas are shown in Table I.

No hepatomas were observed in the mice dying before 36 weeks; at this time 35 of the mice fed the chow diet (3C), and only 24 of those fed the rice diet (3R), were alive. The incidences of hepatomas in the mice alive at the 36th week of the

experiment (and sacrificed by the 45th week, when the experiment was ended) were 46 per cent in group 3C and 42 per cent in group 3R. As in Experiments 1 and 2, the rice diet did not augment the formation of hepatomas.

Experiment 4.—C3H male mice spontaneously develop hepatomas which begin to appear in appreciable number when the mice reach 11 to 12 months of age (1). Each of the two groups in the present experiment was composed of 20 C3H males, 5 to 7 weeks old. The diets employed were the same as those of Experiment 2: Group 4C was fed Purina fox chow checkers *ad libitum*, and group 4R was fed the rice diet. Throughout the experiment, the mice of both groups consumed an average of 3.8 to 4.2 gm. of food daily. The general condition of the mice was excellent, only 1 mouse dying (in 4C) previous to the termination of the experiment when the mice were 13 to 14 months of age. The results are given in Table I.

Again, as in the other 3 experiments, the mice fed the rice diet did not develop more hepatomas than those fed the chow diet. Statistically, there is no difference between the incidence of 5 per cent in the group fed the rice diet, 4R, and the incidence of 18 per cent in the group fed the chow diet, 4C. The brown rice ration supported growth and health as well as Purina fox chow checkers despite its significantly lower protein and riboflavin content.

LIVER LIPIDS

During the period in which *o*-aminoazotoluene was being administered in both Experiments 2 and 3, the total liver lipids of the mice sacrificed for analysis did not vary significantly with the diet. The average total hepatic lipids of the 4 groups ranged from 3.6 to 5.7 per cent of the wet weight of the livers. In the groups fed the chow diets and given the azo dye by subcutaneous injection (group 2C) or by feeding (group 3C), the total liver lipids of individual mice ranged from 3.4 to 5.0 per cent and averaged 4.0 per cent. In the mice of group 2R (rice diet, dye administered by injection) the liver lipids of individual mice ranged from 4.4 to 7.6 per cent, averaging 5.7 per cent; and in group 3R (rice diet, dye fed in the diet) the values ranged from 2.5 to 4.6 per cent, averaging 3.6 per cent.

In contrast, in Experiment 4 (in which *o*-aminoazotoluene was not used) the mice fed the brown rice diet developed mildly fatty livers—containing from 9.0 to 13.1 per cent fat, while those fed Purina fox chow checkers had livers with fat contents from 4.0 to 5.6 per cent.

o-AMINOAZOTOLUENE IN ANIMALS

Before investigating the effects of the diets on the accumulation of *o*-aminoazotoluene in the livers of mice, a few studies were conducted on the distribution and persistence of the dye in the animal body following subcutaneous injection. Only a few representative experiments are given here.

Persistence in tissues following subcutaneous injection.—Twelve C3H male mice were injected subcutaneously with 10 mgm. of *o*-aminoazotoluene; 1, 2, 4, and 6 days later they were sacrificed in groups of 3 mice. The analytical results are given in Table II.

found in the combined injection sites of the individual animals.

In contrast to the other tissues studied, the blood continued to show relatively high levels of *o*-aminoazotoluene for at least 6 days after administration of the dye. As with *p*-aminoazobenzene in blood (17), all the *o*-aminoazotoluene was found in the cells, and none in the plasma. If the method employed for determination of the dye in liver was applied to blood of animals treated with *o*-aminoazotoluene—*i. e.* extraction of the blood with hot acetone and ethyl ether and petroleum ether—none of the dye known to be present was recovered.

TABLE II

	1 day	Micrograms <i>o</i> -aminoazotoluene found at 2 days	4 days	6 days
Liver (per gm.)	7.2–8.4 (7.8)	0.4–2.0 (1.0)	0	0
Blood (per cc.)	7.5–12.2 (10.1)	10.0–14.5 (11.7)	7.1–9.7 (8.5)	6.9–10.2 (8.1)
Urine (per cc.)	16.4–22.0 (18.7)	2.1–8.0 (5.7)	0.8–2.0 (1.4)	0–0.8 (0.3)
Remaining at site of injection	4500–6100 (4900)	1000–3600 (2600)	300–1200 (850)	89–122 (104)

In the many experiments of this kind, appreciable amounts of the dye were found in the livers 20 to 27 hours after the subcutaneous injection of *o*-aminoazotoluene. By 48 hours, the hepatic azo dye was much lower and by 72 hours after injection the levels found were in a range at the limit of sensitivity of the method (about 0.5 μ gm. of dye). This decrease of *o*-aminoazotoluene concentration with time was observed in other tissues such as brain, kidney, and depot fat, but did not obtain for blood.

It is likely that the kidney is a principal route of excretion of not only the metabolic split products (24) but also of the unchanged azo dye. Thus the decreasing concentration of urinary *o*-aminoazotoluene paralleled the disappearance of the azo dye from the tissues and from the injection site.

Relatively rapid disappearance of *o*-aminoazotoluene from the site of injection was consistently observed. During the first 4 days following injection the dye decreased at a rate of about 50 per cent per day. The rate of removal then slowed up so that as late as 1 month following injection of 10 mgm. of *o*-aminoazotoluene 80 to 150 μ gm. of *o*-aminoazotoluene remained at the site of injection. In a number of animals of Experiment 2, sacrificed 1 month after the 7th and final injection of *o*-aminoazotoluene, from 80 to 250 μ gm. of dye was

In order to extract the dye it was necessary to treat the blood with dilute NaOH, as described under Methods. It was also found that blood laked in water and dialyzed against running tap water for 24 hours lost none of the dye. On the other hand, *o*-aminoazotoluene added to blood (laked or whole), of animals which had not been exposed to *o*-aminoazotoluene, could be extracted quantitatively with acetone and ether. These observations suggest that there is formed, *in vivo*, some linkage, salt or otherwise, between *o*-aminoazotoluene and a blood cell component. It also may be inferred that the procedure of extracting with hot acetone and ether permits determination of the *o*-aminoazotoluene in tissues such as liver, kidneys, etc. with assurance that the residual blood in these tissues is not an interfering factor.

Distribution in tissues following subcutaneous injection.—In the following tabulation, the analytical data on two mice, one sacrificed 24 hours and the other 48 hours after injection of 10 mgm. of *o*-aminoazotoluene is given (Table III).

At least in these tissues, the dye appeared to be distributed in proportion to the tissue fat rather than to the total tissue. That even when based on the fat content, the concentration of *o*-aminoazotoluene in the kidneys was consistently high, may

TABLE III: MICROGRAMS OF *o*-AMINOAZOTOLUENE

	24 hours after injection				48 hours after injection			
	Liver	Brain	Kidney	Depot fat	Liver	Brain	Kidney	Depot fat
Per gram of tissue	11	23	21	204	2.2	3.9	3.2	28
Per gram of tissue fat	300	292	400	250	55	49	76	35

be due to the fact that the dye is excreted in the urine. On the other hand, possibly because of a smaller blood supply (6), the fat depots almost always were found to contain less of the dye in relation to the tissue fat.

Effects of the diets on levels of azo dye in the livers.—In Experiment 2, in which the *o*-aminoazotoluene was administered by subcutaneous injection, 6 mice of each group were sacrificed at 24 hours and 6 mice at 48 hours after injection. The range of values for the individual animals, and the average levels of *o*-aminoazotoluene are given in Table IV.

TABLE IV: MICROGRAMS OF *o*-AMINOAZOTOLUENE PER GRAM OF LIVER

Group	24 hours after injection	48 hours after injection
2C-chow diet	1.8–10.3 (6.1)	1.5–5.2 (2.5)
2R-rice diet	5.7–11.0 (8.1)	2.0–4.7 (2.8)

It is apparent that the mice fed the two rations did not differ with respect to the levels of *o*-aminoazotoluene in the livers 24 or 48 hours after injection. Whether differences existed at times later than 48 hours could not be ascertained because the levels observed were too low to be determined with satisfactory precision. The removal of the azo dye from the injection site, the distribution of dye in the tissues other than liver, and excretion in the urine, did not depend upon the diet: the differences between individual mice fed the same diet were so great that the average differences observed between the experimental groups had no significance.

When *o*-aminoazotoluene was fed at a level of 0.05 per cent of the ration as in Experiment 3, the amounts of azo dye found in the livers of individual mice—sacrificed at various times during the day and during the experiment—were about 1 μ gm. or less. In order to obtain reasonably accurate data on the distribution and levels of the dye in such animals it would have been necessary to employ pooled samples of the tissues from 3 or more mice. Nevertheless, such data as were obtained indicated that the dye concentrations in the livers of the mice in the two groups did not differ in a sufficiently consistent way to show any dependence on the diet.

MORPHOLOGY OF THE HEPATOMAS

The hepatomas, both the induced and the spontaneous, observed in these experiments, generally bulged above the hepatic surface as pink to grey, but sometimes yellow or red-brown, globular masses covered by the intact hepatic capsule. Often the induced hepatomas were multiple.

Sections stained with hematoxylin and eosin revealed that the tumors were sharply delineated, often compressing the adjacent hepatic tissue. Both the induced and spontaneous hepatomas were composed of cells which resembled those of the hepatic parenchyma. Although the cells generally were arranged in liver-like cords, there was a lack of true lobular pattern. Often the cells of the tumor would exhibit far fewer degenerative changes, such as fatty infiltration, than those of the liver, but in some instances the reverse was found.

These tumors have been studied by various investigators (3, 10, 25) and the question has arisen as to whether they should be regarded as extreme examples of active non-architectural regeneration or as tumors. Certainly there seems to be no good clinical or morphologic reasons to consider them malignant. A careful evaluation of the studies of others, and our own, suggests that the mouse hepatomas—either those induced by *o*-aminoazotoluene or those occurring spontaneously (as in the C3H strain)—should be considered benign tumors.

DISCUSSION

In the four experiments concerned with hepatoma formation in mice, the animals fed rice diets developed either the same or a lower incidence of hepatomas than the mice fed the more adequate chow diets. In none of the experiments did the mice fed the rice diets develop a higher percentage of hepatomas. This experience is contrary to that which might be expected since such rice diets, in comparison with more adequate rations, effect a marked acceleration of liver tumor formation in rats fed *p*-dimethylaminoazobenzene.

In the three experiments with induced hepatomas, the unexpected failure of the rice diet to accelerate hepatoma formation in mice exposed to *o*-aminoazotoluene may be due to (a) the difference in carcinogen—*o*-aminoazotoluene was employed as the carcinogen in these studies while most of the

definitive dietary work with rats has been done with *p*-dimethylaminoazobenzene, or (b) the difference in species—the mouse responding differently from the rat.

The nature and extent of the modifying effect of diet on tumor formation is dependent on the particular carcinogenic agent utilized. Liver extract or increased dietary protein are reported to have no demonstrable effect on the formation of hepatomas induced by 2-acetylaminofluorene fed to rats (8); and riboflavin, hydrogenated coconut oil, or rice bran in the diet have a smaller and less consistent effect on tumor formation when *m*'- or *o*'-methyl-*p*-dimethylaminoazobenzene is employed to induce tumors than occurs with *p*-dimethylaminoazobenzene (7). In rats receiving *o*-aminoazotoluene, however, tumor formation is definitely modified by the dietary regimen. For example, rats fed rice containing the dye for about 8 months developed a high incidence of hepatic tumors, while no liver tumors were found in rats fed wheat containing the same amount of the dye for as long as one year (5, 29); 8 of 24 rats fed rice (and 0.02 to 0.1 per cent *o*-aminoazotoluene) for 1 year developed hepatomas, in contrast to only 1 of 38 rats fed the rice diet with 10 per cent liver powder and the same amounts of dye (18). Similarly, Maisin (11) failed to observe any hepatomas in 100 rats fed *o*-aminoazotoluene in a diet of wheat, wheat germ, and beef for 700 days, in sharp contrast to the results obtained by workers feeding the dye in a rice diet. Thus it is probable that hepatomas would form more rapidly in rats fed *o*-aminoazotoluene in a rice diet than in rats given the dye in a ration of fox chow alone or with added milk powder and cornstarch.

On the other hand, the diversity of the effects in mice and rats may be due to species differences in general, or more specifically to the difference in the nature of the types of liver tumors produced in the two species by the azo dyes. In the mouse the hepatomas, induced by *o*-aminoazotoluene or occurring spontaneously, are *benign* tumors the cells of which fundamentally resemble the normal parenchymal cells of the liver. On the other hand, in the rat the hepatomas induced by *p*-dimethylaminoazobenzene and other azo dyes are varied in type—bile-duct carcinoma and cystadenoma, liver cell carcinoma, and occasionally benign hepatoma—but the majority are *malignant* tumors (20, 21). It may be that the carcinogenic process involved in the production of benign hepatoma in the mouse is affected by dietary changes in an entirely different way than the carcinogenic process involved in the

production of the malignant tumors in the rat.

The original object in performing analyses for hepatic lipids and *o*-aminoazotoluene was based on the anticipation of a striking dietary effect on hepatoma formation. Since the differences in hepatoma formation were not in the direction expected, and not of great magnitude, the information gained from such analyses is rather limited. No differences were observed between animals on the two types of diet with respect to the levels of *o*-aminoazotoluene in the liver, the distribution of the dye in the tissues, or the rate of removal of the dye from the site of injection. Nor were there any consistent differences in total liver lipids. These results were obtained in Experiments 2 and 3 in which the differences in incidences of hepatomas also were small. Consequently, the present experiments do not give any information on whether dietary effects on the formation of hepatomas induced in mice by azo dyes are mediated through an effect on hepatic lipids or on levels of carcinogen present in the liver.

That the incidence of spontaneously occurring hepatomas in C3H male mice is not augmented, but possibly depressed by the rice diet (Experiment 4), is in agreement with experiments indicating that a low protein (9 per cent casein) diet, sufficient to support growth and body health, retards the formation of spontaneous hepatomas in mice as compared with otherwise similar diets containing 18 or 45 per cent casein (28). It does not seem likely at present that the increased level of hepatic lipids observed in the mice fed the brown rice diet was of any significance in the possibly depressed hepatoma formation.

In the rat, the rate of formation of hepatic tumors induced by various carcinogens is known to be dependent on diet. The dependency on diet, however, appears to be modified by the nature of the carcinogen (7, 8). There is suggestive evidence that diets control hepatoma formation in rats through an effect on the levels of hepatic riboflavin (13), or on the levels of the carcinogen or its derivatives in the liver (12, 15, 23), although they may be only associated changes rather than causative factors. The experiments reported in this paper indicate that the species of animal (or perhaps the type of tumor induced) may be another factor, controlling the degree and even the direction of the change in hepatoma formation produced by dietary means.

SUMMARY

Mice fed diets composed essentially of rice developed hepatomas at the same or a slower rate

than mice fed a more adequate commercial diet containing higher amounts of protein, riboflavin, and other essential components. This was observed in two experiments in which hepatomas were induced by injection of *o*-aminoazotoluene, in one experiment in which hepatomas were induced by the feeding of *o*-aminoazotoluene, and in another experiment in which the spontaneously occurring hepatoma of the C3H male mouse was employed.

This observation, that a rice diet did not accelerate the formation of hepatomas in mice, is in contrast to the well-established fact that comparable rice diets do accelerate the formation of hepatomas, induced by *p*-dimethylaminoazobenzene in rats. It is suggested that this difference between mouse and rat may be a species difference—particularly related to the fact that the hepatomas induced in the mouse are benign while those induced in the rat are, in the main, malignant.

ACKNOWLEDGMENT

This work was done under the direction of Dr. T. F. Gallagher, Department of Biochemistry, University of Chicago, and Dr. Albert Tannenbaum, Department of Cancer Research, Michael Reese Hospital.

REFERENCES

- ANDERVONT, H. B. Spontaneous Tumors in a Subline of Strain C3H Mice. *J. Nat. Cancer Inst.*, **1**:737-744. 1941.
- ANDERVONT, H. B., and EDWARDS, J. E. Carcinogenic Action of Two Azo Compounds in Mice. *J. Nat. Cancer Inst.*, **3**:349-354. 1943.
- ANDERVONT, H. B., and GRADY, H. G. Induction of Hepatic Lesions, Hepatomas, Pulmonary Tumors, and Hemangio-Endotheliomas in Mice with *o*-Aminoazotoluene. *J. Nat. Cancer Inst.*, **3**:131-153. 1942.
- ANDERVONT, H. B., WHITE, J., and EDWARDS, J. E. Effect of Two Azo Compounds When Added to the Diet of Mice. *J. Nat. Cancer Inst.*, **4**:583-586. 1944.
- ANDO, T. Einfluss der Futtermittel auf die experimentelle Leberkarzinomenstehung. Experimentelle Leberkarzinomenstehung mit getreide (Zeitdauer d. Fütterung) *Gann*, **34**:371-373. 1940.
- GERSH, I., and STILL, MARY A. Blood Vessels in Fat Tissue. Relation to Problems of Gas Exchange. *J. Exper. Med.*, **81**:219-232. 1945.
- GIESE, J. E., CLAYTON, C. C., MILLER, E. C., and BAUMANN, C. A. The Effect of Certain Diets on Hepatic Tumor Formation Due to *m'*-Methyl-*p*-Dimethylaminoazobenzene and *o'*-Methyl-*p*-Dimethylaminoazobenzene. *Cancer Research*, **6**:679-684. 1946.
- HARRIS, P. N. Production of Tumors in Rats by 2-Aminofluorene and 2-Acetylaminofluorene. Failure of Liver Extract and of Dietary Protein Level to Influence Liver Tumor Production. *Cancer Research*, **7**:88-94. 1947.
- KINOSITA, R. Studies on the Cancerogenic Azo and Related Compounds. *Yale J. Biol. & Med.*, **12**:287-300. 1940.
- LIPPINCOTT, S. W., EDWARDS, J. E., GRADY, H. G., and STEWART, H. L. A Review of Some Spontaneous Neoplasms in Mice. *J. Nat. Cancer Inst.*, **3**:199-210. 1942.
- MAISIN, J. La prophylaxie du Cancer. Étude expérimentale. *Bull. Assoc. franc. p. l'étude du Cancer*, **29**:6-23. 1940.
- MILLER, ELIZABETH C., and MILLER, J. A. The Presence and Significance of Bound Aminoazo Dyes in the Livers of Rats Fed *p*-Dimethylaminoazobenzene. *Cancer Research*, **7**:468-480. 1947.
- MILLER, J. A. Studies on the Mechanism of the Effects of Fats and Other Dietary Factors on Carcinogenesis by the Azo Dyes. *Ann. N.Y. Acad. Sc.*, **49**:19-28. 1947.
- MILLER, J. A., and BAUMANN, C. A. The Determination of *p*-Dimethylaminoazobenzene, *p*-Monomethylaminoazobenzene, and *p*-Aminoazobenzene in Tissue. *Cancer Research*, **5**:157-161. 1945.
- MILLER, J. A., KLINE, B. E., and RUSCH, H. P. The Inhibition of the Carcinogenicity of *p*-Dimethylaminoazobenzene by Certain Detergents and the Effect of Diet on the Levels of Azo Dyes in Rat Tissues. *Cancer Research*, **6**:674-678. 1946.
- MILLER, J. A., MINER, D. L., RUSCH, H. P., and BAUMANN, C. A. Diet and Hepatic Tumor Formation. *Cancer Research*, **1**:699-708. 1941.
- MILLER, J. A., MILLER, E. C., and BAUMANN, C. A. On the Methylation and Demethylation of Certain Carcinogenic Azo Dyes in the Rat. *Cancer Research*, **5**:162-168. 1945.
- MORI, K. Effect of Liver Feeding on Liver Cancer Production by *o*-Aminoazotoluol. *Gann*, **35**:106-119. 1941.
- NAKAHARA, W., MORI, K., and FUJIWARA, T. Inhibition of Experimental Production of Liver Cancer by Liver Feeding. A Study in Nutrition. *Gann*, **33**:406-428. 1939.
- OPIE, E. L. The Pathogenesis of Tumors of the Liver Produced by Butter Yellow. *J. Exper. Med.*, **80**:231-246. 1944.
- ORR, J. W. The Histology of the Rat's Liver During the Course of Carcinogenesis by Butter-Yellow (*p*-Dimethylaminoazobenzene). *J. Path. & Bact.*, **50**:393-408. 1940.
- RUSCH, H. P., BAUMANN, C. A., MILLER, J. A. and KLINE, B. E. Experimental Liver Tumors. A.A.A.S. Research Conference on Cancer. Gibson Island, Washington, D.C. 1945, pp. 267-287.
- SILVERSTONE, H. The Levels of Carcinogenic Azo Dyes in the Livers of Rats Fed Various Diets Containing *p*-Dimethylaminoazobenzene. Relationship to the Formation of Hepatomas. *Cancer Research*, **8**:301-308. 1948.
- STEVENSON, ELIZABETH S., DOBRINER, K., and RHOADS, C. P. The Metabolism of Dimethylaminoazobenzene (Butter Yellow) in Rats. *Cancer Research*, **2**:160-167. 1942.

25. STRONG, L. C., and SMITH, G. M. Benign Hepatomas in Mice of the CBA Strain. *Am. J. Cancer*, **27**:279-284. 1936.
26. SUGIURA, K., and KENSLER, C. J. Experimental Liver Cancer and Its Inhibition by Various Food Substances. Abstr., American Association for Cancer Research, Inc., 34th Annual Meeting, Chicago, Ill., April 14-16. *Cancer Research*, **1**:745-746. 1941.
27. SUGIURA, K., and RHOADS, C. P. Experimental Liver Cancer in Rats and Its Inhibition by Rice Bran Extract, Yeast, and Yeast Extract. *Cancer Research*, **1**:3-16. 1941.
28. TANNENBAUM, A., and SILVERSTONE, H. Effect of Varying the Protein (Casein) Content of the Diet on the Formation of Tumors in the Mouse. Abstr., American Association for Cancer Research, Inc., 38th Annual Meeting, Chicago, May 16 and 17, 1947. *Cancer Research*, **7**: 711. 1947.
29. VASSILIADIS, H. C. Carcinogenic Properties of Ortho Amidoazotoluol. *Am. J. Cancer*, **39**:377-378. 1940.
30. WESSON, L. G. A Modification of the Osborn-Mendel Salt Mixture Containing Only Inorganic Constituents. *Science*, **75**:339-340. 1932.

Comparative Studies of the Immunological, Toxic and Tumor-Necrotizing Properties of Polysaccharides from *Serratia marcescens* (*Bacillus prodigiosus*)*

Hugh J. Creech, Ph.D., Mary Alice Hamilton, Ph.D.,** and Irene Corey Diller, Ph.D.

(From the Lankenau Hospital Research Institute and the Institute for Cancer Research, Philadelphia 30, Pa.)

(Received for publication December 10, 1947)

These studies are concerned with the immunological, toxic and tumor-necrotizing properties of purified polysaccharide-lipid complexes obtained by the methods of Shear and his associates (8, 14) from filtrates of cultures of *Serratia marcescens* (*Bacillus prodigiosus*). The project was undertaken as part of a joint institutional program with the Chemotherapy Section of the National Cancer Institute.

The preparations supplied to us by Dr. M. J. Shear were effective in causing necrosis in mouse sarcoma 37. Although this effect on primary and transplanted tumors has been studied by Shear and his group (12, 13) at the National Cancer Institute and by Diller (5, 6) at this Institute, little investigation has been made of the immunological properties of purified preparations of these polysaccharides. Schwartzman (11) found 2 of Shear's earlier lyophilized preparations of polysaccharide to be antigenic in rabbits. He concluded from his experiments that the active principles in the culture filtrates responsible for local skin reactivity had been concentrated and purified without inducing any measurable alteration in their antigenicity. Schwartzman also stated that the principles causing local skin reactivity were closely related to or identical with the factors capable of inducing hemorrhages and regressions of mouse tumors. It has been suggested (12) that an immunological mechanism may be responsible for the general observation that repeated injections of polysaccharide are less effective than the first injection in causing hemorrhage and necrosis of sarcomas and in producing toxic symptoms. On the other hand, Franke (7) found that guinea pigs sensitized to a lyophilized preparation of *Serratia marcescens* polysac-

charide did not exhibit anaphylactic shock following the second dose of polysaccharide.

A major point of interest in our studies has been the determination of whether the antigenic, toxic and tumor-necrotizing properties always run parallel in various preparations of the polysaccharide. Observations of the toxic and tumor-necrotizing actions have been made on mice and rabbits. Immunological studies have been conducted on normal rabbits and on patients with various types of tumors.

EXPERIMENTAL

PREPARATIONS OF POLYSACCHARIDES

The preparations, supplied to us as solutions containing from 2.5 to 10 mgm. of polysaccharide per cc., were of 2 main types (a) *P-3*, which was obtained from cultures of *Serratia marcescens* of strain G.W. and (b) *P-5* and *P-10*, which were obtained from strain 724.¹ Among the *P-3* types, there were 3 lots which have been designated as follows: *P-3.0*, a preparation which had been preserved with phenol and stored at room temperature, *P-3.N*, a preparation which had been preserved with toluene and stored also at room temperature and *P-3.S* which had been preserved with toluene and stored at 10°. In addition, studies were made with *P-3.F* a supply of polysaccharide which accidentally had been frozen while in solution. All of these preparations came from the same original batch of polysaccharide prepared about 5 years ago.

Because of the shortage of the *P-3* type of polysaccharide during the earlier part of this study, a collection was made of all the residues of various dilutions left over from experiments conducted in this Institute during the past few years. The polysaccharide was precipitated by adjusting the pooled solutions to pH 2 to 3 and adding 3 volumes of

* Presented in part at the 38th Annual Meeting of the American Association for Cancer Research, Chicago, May 16 and 17, 1947.

** Present address: The Colorado Foundation for Research in Tuberculosis, Colorado College, Colorado Springs, Colorado.

¹The cultures from which *P-5* and *P-10* were made were grown at the Lederle Laboratories; isolation and purification of the polysaccharides were done by Perrault and Shear.

ethyl alcohol and 2 volumes of acetone. The precipitate, after removal by centrifugation, was dissolved in physiologic saline and dialyzed in turn against running tap water, distilled water, and physiologic saline. A small amount of insoluble material was removed by centrifugation and filtration. An aliquot of the solution was treated with alcohol and acetone to determine the amount of polysaccharide per cc. The solution was designated *P-3.Ra*.

Fractionation of several lots of frozen polysaccharide yielded some interesting products. One fraction, designated *P-3.Rb*, was obtained from a 10 mgm. per cc. solution of polysaccharide by precipitation at pH 2 to 3 with 3 volumes of ethyl alcohol and $1\frac{1}{2}$ volumes of acetone. The precipitate was removed by centrifugation and dissolved in 1 volume of 0.85 per cent sodium chloride solution containing sufficient sodium hydroxide to adjust the pH to 9. The addition of 3 volumes of alcohol to this alkaline solution caused the precipitation of no more than a trace of insoluble material which was removed by centrifugation. As no further precipitation was caused by the addition of $1\frac{1}{2}$ volumes of acetone, the solution was acidified with hydrochloric acid to adjust the pH to 2 to 3. The resultant, almost colorless, precipitate was removed by centrifugation and dissolved in $\frac{1}{2}$ volume of 0.85 per cent sodium chloride solution. This solution was dialyzed against running tap water for 8 hours and then against several changes of cold 0.85 per cent sodium chloride solution, buffered at pH 7.5 with phosphate, for 30 hours. The solution was diluted with physiologic saline to the 2-volume level, centrifuged, and filtered in the refrigerator through Whatman No. 1 paper. By precipitation of an aliquot of the solution with alcohol and acetone, followed by washing of the precipitate with acetone and drying in the oven at 50°, the amount of isolated polysaccharide indicated a 50 per cent recovery of the original amount present in the solution of frozen polysaccharide.

In another case, the polysaccharide was precipitated from a neutral solution of formerly frozen polysaccharide containing 10 mgm. per cc. by the addition of 4 volumes of ethyl alcohol and 2 volumes of acetone. To the precipitate were added 2 volumes of 0.85 per cent sodium chloride solution, 1 volume of ethyl alcohol and 1 volume of acetone. The turbid solution was adjusted to pH 9 and allowed to stand in the refrigerator for 1 hour. The gelatinous portion (A) was separated from the soluble material (B) by centrifugation. The soluble portion (B) was acidified to pH 2 to

3 and the polysaccharide was precipitated by the addition of alcohol and acetone in the usual way. The precipitated material was dissolved in physiologic saline buffered at pH 7.5 and dialyzed as usual. Analysis showed that the final solution contained 24 per cent of the polysaccharide present in the original preparation. This fraction was designated *P-3.Rb-3*. The material (A), which was insoluble at pH 9, was suspended in $\frac{1}{2}$ volume of 0.85 per cent sodium chloride solution buffered at pH 7.5 and dialyzed against the same solvent. During dialysis, most of the material went into solution. Upon analysis, the centrifuged solution was found to contain 60 per cent of the polysaccharide present in the original preparation. This fraction was designated *P-3.Rb-4*.

By somewhat similar simple procedures, several other fractions were obtained. Since these did not exhibit characteristics significantly different from those of the starting material, no description of their preparation or properties has been recorded in this paper. A sample of P-5 solution was frozen intentionally; the product was designated *P-5.F*.

There are considerable differences in the nitrogen content of some of the original preparations as reported by Dr. Shear. The values are as follows: P-3.N, 1.4 per cent; P-5, 1.4 per cent; P-10, 2.4 per cent.

To aid the reader in interpreting the tables of results, the various preparations of polysaccharides with their symbols are listed in Table I.

TABLE I: LIST OF POLYSACCHARIDES

Symbol	Strain from which obtained	Additional description
P-3.0	G. W.	stored at 25° with phenol
P-3.N	G. W.	" " 25° " toluene
P-3.S	G. W.	" " 10° " toluene
P-3.F	G. W.	accidentally frozen
P-3.Ra	G. W.	fractionated from P-3
P-3.Rb	G. W.	" " P-3.F
P-3.Rb-3	G. W.	" " P-3.F
P-3.Rb-4	G. W.	" " P-3.F
P-5	724	N content 1.4%
P-5.F	724	intentionally frozen
P-10	724	N content 2.4%

PROCEDURES OF IMMUNIZATION

The method adopted was based on Shwartzman's observations (11) that a satisfactory degree of immunization of rabbits was accomplished by relatively large, intramuscular injections of the polysaccharide. Accordingly, 4 intramuscular injections of 0.25 to 0.5 cc. containing approximately 500 γ of polysaccharide per 3 kgm. of body weight in physiologic saline, were given at 3 to 4 day intervals alternately in the hind legs of the rabbits. This

amount of some of the preparations was found to be lethal; consequently, with such preparations, it was necessary to start with doses of 10 or 100 γ and to increase the amount gradually to 500 γ , giving in all about 2 mgm. of the polysaccharide over a period of 2 to 4 weeks. In some cases, intravenous booster injections of 50 or 100 γ of polysaccharide in 0.1-0.25 cc. of physiologic saline were given after the intramuscular series. In an attempt to obtain stronger antisera (2) toward certain preparations, 2 cc. of Upjohn aqueous adrenal cortical extract was administered subcutaneously. When the antibody titer had reached a satisfactory level, the rabbits were exsanguinated by cardiac puncture. About 7 cc. of antiserum from each rabbit was frozen and stored; the remaining portions of the antisera were pooled according to type.

FRACTIONATION OF ANTISERA

The pooled antisera toward the various preparations of polysaccharide were fractionated at 10° by equilibration with ammonium sulfate using the rotating cellophane membrane technic (10). The first fraction was obtained by precipitation with this reagent within the range of 1.0 to 1.5 molar, a second fraction within the 1.5 to 2.1 molar range and a third within the 2.1 to 2.5 molar range of ammonium sulfate solution. The main protein components separating in these ranges were γ -globulin for the first, α - and β -globulins for the second, and albumin for the third range. The globulin fractions were refractionated within narrower limits of ammonium sulfate concentration. The fractions were stored at 5 to 10° under 2 *M* ammonium sulfate solution. When required for tests, a portion was dialyzed against running tap water, distilled water, and 0.85 per cent sodium chloride solution at 10°. The protein content of the solution was determined by micro-Kjeldahl analysis.

SEROLOGICAL TESTS

(a) *Precipitin tests*.—After preliminary determinations of the amount of polysaccharide test antigen needed for satisfactory serological tests, the standard procedure involved the use of a constant small amount of polysaccharide and serial dilutions of the antisera. The potencies of the antisera were determined by layering 50 γ of polysaccharide in 0.2 cc. of saline over 0.2 cc. of serial dilutions of antisera ranging from undiluted to a dilution of 1:512. Tubes of 5 mm. inside diameter were used and the layering was done with Tuberculin syringes and 2-inch, 18-gauge needles. The rings forming at the junction of the 2 layers were read in the

standard manner and given values ranging from trace to 4 plus. The best readings normally were obtained 15 minutes after the overlaying was made; in the case of weaker antigens, more satisfactory readings were obtained after an additional hour, also at room temperature.

(b) *Complement fixation tests*.—An adaptation of Kolmer's technic (9) was employed. Commercial preparations of hemolysin and of lyophilized complement were used; the sheep red cells were obtained from the School of Veterinary Medicine, University of Pennsylvania. Buffered physiologic saline of pH 7.4 was used throughout as the diluting medium. Hemolysin and complement were titrated at the beginning of each run. Two exact units of hemolysin, two full units of complement and a well-washed 2 per cent suspension of sheep cells were employed in the tests. Each lot of polysaccharide, except P-10, was titrated against serial dilutions of a pool of positive antisera to determine the antigenic unit. The antigenic dose, which was 8 times the antigenic unit, ranged from 1 to 50 γ per 0.5 cc.

Primary and secondary incubations were 1 hour in a 37° water bath. The initial reading was made when the antigenic and hemolytic controls were hemolyzed completely. The next reading was made at the end of the hour of secondary incubation and the 2 readings were averaged. The end point was considered to be the highest dilution which gave readings averaging more than 1 plus. Serum, antigen, cell and hemolytic system controls were run for each series of tests. Rabbit antisera were inactivated at 63° for 5 minutes and tested in dilutions of 1:40, 1:80 up to 1:640. Human antisera were inactivated at 56° for 30 minutes and tested in dilutions of 1:2.5, 1:5 up to 1:100.

(c) *Agglutinin tests*.—A heat-killed suspension of *Serratia marcescens*, strain 724, was used in the tests. One-half cc. of suspension containing approximately 1 billion organisms per cc., as determined by comparison with barium sulfate standards, was mixed with 0.5 cc. of antiserum diluted 1:5, 1:10, 1:20, etc. The mixtures were incubated for 2 hours at 37° and then allowed to stand in the refrigerator overnight. The usual antigen control was run with each series of tests. The end point was considered the highest dilution which gave at least a \pm reading.

In all cases, serological tests were conducted on rabbits and on patients before the administration of polysaccharide to ensure that no positive reactions were given prior to the injections. Only 1 of the 60 rabbits showed a reaction before the ad-

ministration of polysaccharide; none of the patients was positive prior to polysaccharide treatment.

LETHAL ACTIVITY

The preparations of polysaccharide were assayed for their lethal activity by determining the mortality resulting from the injection of large amounts of the materials into normal mice. Whenever enough of a preparation was available, attempts were made to find the level that would kill 70 to 90 per cent of the mice within 24 to 48 hours. Preliminary studies were made on mixed breeds of mice of ages varying from 2 to 8 months (designated mx in Table VI). Later studies were conducted on mice about 2 months of age. "A" strain mice, a strain of Swiss mice, and mice resulting from the mixed breeding of descendants of a cross between C and A strain mice (designated CAF in

Table VI) were used in these studies. The polysaccharides were dissolved either in 0.25 cc. or in 0.5 cc. of saline and injected by the intraperitoneal route.²

STUDIES OF TUMOR-NECROTIZATION

In order to compare the tumor-necrotizing potencies of the various preparations of polysaccharide, a 10- or 20- γ quantity in 0.1 or 0.2 cc. of saline

²Merthiolate (Lilly) was added to the stock solutions of polysaccharides, after dialysis to free them of other preservatives, to give a final concentration of 1 : 10,000. There has been no indication that the use of merthiolate has had any influence either on the toxicity or tumor-necrotizing action of the polysaccharides. Since we had the impression that the tumor-necrotizing action of the polysaccharide decreased on long-standing even in concentrated solutions, the required amounts of diluted solutions used both for toxicity and tumor-necrotizing tests were freshly prepared from stock solutions a few hours before use.

TABLE II: COMPLEMENT-FIXATION REACTION

Test antigens

Rabbit no.	Method† imm.	Antiserum toward	P-3.0 Amt. in γ 1	P-3.N 1	P-3.S 1	P-3.F 1	P-3.Ra 4	P-3.Rb 20	P-3. Rb-3 5	P-3. Rb-4 1.3
7	b	P-3.0	320	320	320	640	320	320	160	160
10	b		80	80	80	160	80	80	40	40
11	b		640	640	640	640	640	640	320	640
66	a		160	160	160	320	160	160	160	80
4	b	P-3.N	80	160	80	160	80	80	40	40
5	b		320	320	320	320	320	320	320	320
6	b		320	320	320	640	320	160	80	160
17	c	P-3.S	160	160	160	320	160	80	40	80
18	c		80	80	80	160	80	80	40	40
22	d		320	320	320	640	160	160	80	160
23	d		320	320	640	640	320	320	80	160
28	a	P-3.F		320	320		160			
36	e		320	320	320	640	320	320	640	640
37	e		320	320	320	640	320	320	640	320
38	e		320	320	160	640	320	320	320	160
3*	a	P-3.Ra	640	640	640	640	640	640	640	640
8	b		80	80	80	160	80	80	40	80
14	b		320	320	320	640	320	320		
29	g	P-3.Rb	<40	<40	<40	<40	<40	<40	<40	<40
30	h		<40	<40	<40	<40	<40	<40	<40	<40
32	i		40	40	40	40	40	<40	<40	<40
33	f		160	160	80	80	160	80	160	80
39	a	P-3.Rb-3	320	160	160	320	160	160	160	80
40	a		<40	<40	<40	<40	<40	<40	<40	<40
34	j	P-3.Rb-4	640	640	640	640	640	640	640	640

* This rabbit gave positive reactions prior to immunization

† Method of Immunization

- Four intramuscular injections of 500 γ each.
- Four intramuscular injections of 500 γ each plus a 500 or 100 γ booster i.v.
- Intramuscular injections as follows: 20, 20, 20, 20, 50, 100, 200, 300, 400, 500, 500 γ .
- Intramuscular injections as follows: 300, 300, 300, 400, 400, 500 γ .
- Intramuscular injections as follows: 100, 200, 200, 200, 300, 500, 500 γ , plus a 50 γ booster i.v.
- Same as (b), plus 5 daily injections of 2 cc. of adrenal cortical extract subcu. and 50- γ booster i.v.
- Same as (b), plus 10 cc. of adrenal cortical extract subcu. plus a 100 γ booster i.v.
- Same as (b), plus 10 cc. of adrenal cortical extract subcu.
- Same as (b), plus a 100 γ booster i.v.
- Intramuscular injections as follows: 100, 200, 300, 400, 500, 500 γ .

was injected intraperitoneally into Swiss mice bearing sarcoma 37 dorsal implants which had grown to a diameter of 8 to 10 mm. Tumors were removed for cytological study at 6 hours, 24 hours and 3 days post-injection. The techniques employed have been described in detail by Diller (6). In certain instances, experiments also were made to determine the number of tumor regressions caused by the injection of the polysaccharide.

RESULTS

IMMUNOLOGICAL RESULTS

Because of the limited supply of certain polysaccharides, the complement-fixation technic was employed as the main method for comparing the antisera produced by the injection of the various P-3 type polysaccharides into rabbits. The results of these tests are presented in Table II. The figures in the table represent the reciprocal of the greatest dilution of antiserum giving the characteristic end point. Across the top of the table are listed the antigenic doses in terms of γ of the polysaccharide used as test antigen. From these figures, it may be seen that 1 γ of polysaccharide was sufficient in the case of the preparations P-3.O, P-3.N, P-3.S and P-3.F, whereas the 4 fractionated products were less effective as test antigens and had to be used in greater amounts. With regard to the levels of antibody in the various antisera, it will be noted, as is found generally, that some rabbits produced higher antibody levels than other rabbits in the same series. In general, the preparations P-3.O, P-3.N, P-3.S and P-3.F, produced equally strong antisera which reacted equally well with all of the test antigens P-3.O, P-3.N, P-3.S and P-3.F. The P-3.F preparation, however, was a slightly more sensitive test antigen. It was impossible, therefore, to differentiate among these antisera or antigens by serological methods. Storage of these preparations under the different conditions seemed to have no appreciable effect on their antigenic activity.

Products obtained by fractionation of the P-3 polysaccharide, however, had different antigenic activities. Thus, P-3.Ra elicited good antisera which reacted well with all the various types of polysac-

charides. This preparation also reacted well with the various types of antisera although the test antigenic dose of P-3.Ra was larger than that of the original preparations. With the use of P-3.Rb, much weaker antisera were produced and an even greater test antigenic dose (20 γ of P-3.Rb) was required in tests with the various antisera. The fraction P-3.Rb-4 appeared to have the same antigenicity as the original preparations. It was highly toxic also as it killed 2 of the 3 rabbits at the initial 500- γ intramuscular injection. The product P-3.Rb-3, which was separated from P-3.Rb-4 at the final stage of fractionation, appeared to be a poorer antigen. Unfortunately, the lack of sufficient amounts of these two products to immunize additional rabbits prevented adequate study of their immunizing capacity.

These observations are supported by the results of the precipitin tests. Only the 2 most interesting series of antisera are considered in Table III. The test antigenic level was 50 γ of each polysaccharide. Antisera toward the P-3.F preparation reacted strongly with all the original preparations of polysaccharide as is shown by the figures which represent the reciprocal of the greatest dilution of antiserum which gave a \pm reaction in the precipitin tests. These antisera did not react well with the P-3.Rb polysaccharide which, therefore, can be considered to be a weak test antigen. It is apparent also that P-3.Rb was not effective in stimulating strong antibody formation. Precipitin tests with antisera toward P-3.O, P-3.N and P-3.S (not recorded herein) fell in the range not exceeding a 1:32 dilution of antiserum and hence these antisera were intermediate between P-3.F and P-3.Rb in antibody stimulating potency as judged by this test.

The results of comparative studies of antisera toward P-3.F, from the G.W. strain of *Serratia marcescens*, and of antisera toward P-5 and P-10, both of which came from the 724 strain, are recorded in Table IV. There appears to be no apparent serological relationship between the polysaccharides from the 2 different strains. The antisera toward any one type did not react significantly

TABLE III: PRECIPITIN REACTIONS

Rabbit no.	Method imm.	Antiserum toward	Test antigens (50 γ)					
			P-3.O	P-3.N	P-3.S	P-3.F	P-3.Ra	P-3.Rb
36	e	P-3.F	256	128	256	128	256	4
37	e		128	64	64	64	128	8
38	e		256	128	256	128	128	8
29	g	P-3.Rb	4	4	4	2	4	-
30	h		2	-	1	2	2	2
32	i		-	1	1	1	2	2
33	f		8	4	4	2	4	-

TABLE IV: PRECIPITIN, AGGLUTININ AND COMPLEMENT-FIXATION REACTIONS

Rabbit no.	Method imm.*	Antiserum toward	Test antigens						Strain 724 Agg. 5 x 10 ⁸ bac.
			Test c.f. amt. γ 1	P-3.F pptn. 50	c.f. 50	P-5 pptn. 50	c.f. 50	P-10 pptn. 50	
73	a	P-3.F	640	128	—	—	—	—	160
74			640	128	—	—	—	—	—
75			640	128	—	—	—	—	40
76			640	128	—	—	—	—	—
68	b	P-5	—	—	<40	1	<40	—	160
69			—	—	80	2	80	—	640
70			<40	—	<40	—	<40	—	20
71			—	—	<40	—	<40	—	40
77	b+c	P-5ach	—	—	640	1	160	2	2560
78			—	—	640	2	640	2	5120
79			—	—	320	2	320	2	5120
80			<40	—	160	4	160	2	1280
81	a	P-10	—	—	160	32	160	8	—
82			—	—	320	16	640	16	—
83			—	—	320	16	640	8	—
84			—	—	640	16	640	16	—
81	a+c	P-10ach	—	—	80	16	160	16	640
82			—	—	160	16	160	16	1280
83			—	—	160	32	80	16	640
84			—	—	160	16	320	16	2560

* (a) 6 intramuscular injections of 100, 200, 300, 400, 500 γ per 3 kgm. of body weight every third day.

(b) 4 intramuscular injections of 500 γ per 3 kgm. of body weight every third day.

(c) 5 subcutaneous injections of 2 cc. of adrenal cortical extract and 5 intravenous injections of 50 γ of polysaccharide on successive days.

with the other type polysaccharide in any of the 3 serological tests. The P-3.F preparation was far more antigenic than either the P-5 or the P-10 preparation. The P-10 preparation, however, was a more effective stimulator of antibodies than P-5. In the case of P-5, the use of booster injections of P-5 and of adrenal cortical extract aided considerably in the production of stronger antisera (listed as P-5 ach). There was no pronounced alteration in the precipitin tests, possibly because of the lower sensitivity of this test, but the complement-fixation results increased from almost insignificant values with P-5 antisera to relatively high values with antisera toward P-5 when antibody production was aided by booster shots and adrenal cortical hormone injections.³

It will be noted in the complement-fixation tests that P-5 and P-10 were only moderately effective test antigens because an amount of 50 γ seemed

necessary whereas, with P-3.F, only 1 γ was needed. The test antigen P-10 reacted equally as well as P-5 with the antisera toward both P-5 and P-5 ach. In the agglutination tests with strain 724 organisms, the antisera toward P-5 ach gave much stronger reactions than those toward P-5 alone.

The serological results with antisera toward P-10 were obtained on samples of blood withdrawn from the rabbits 10 days after the final intramuscular injection. These rabbits then were given booster shots of P-10 along with adrenal cortical extract and were exsanguinated within 6 hours after the last of these injections. The latter sera are designated as antisera toward P-10 ach. The P-10 preparation alone, unlike P-5, stimulated the formation of relatively strong antisera which reacted equally well with both the P-10 and P-5 test antigens. The booster injections of P-10 and the adrenal cortical extract seemed to have no beneficial influence on the antibody content of the rabbit sera. Thus it appears that P-5 and P-10 as test antigens are essentially indistinguishable one from the other; P-5 by itself is extremely weak in its ability to produce antisera whereas P-10 is relatively effective in this respect. In the agglutinin tests, antisera toward P-5 ach seemed to be more effective than the other antisera.

These observations lead to the following conclusions: (a) that there is no apparent serological

³Serological tests, made recently in this Department by Dr. D. R. A. Wharton, indicated that the use of undiluted antiserum and successively diluted test antigen occasionally caused more satisfactory precipitin reactions than the alternative method used in the above work. This was the case particularly with polysaccharides P-3.Rb and P-5 and their antisera. The conclusions about the relative weakness in antibody-stimulating activity of these two polysaccharides remain unaltered; there also was no significant indication of any serological relationship between the polysaccharides from the two different strains of *Serratia marcescens*.

relationship between the two types of polysaccharide from the different strains of *Serratia marcescens*, (b) that the preparations, P-5 and P-10 from strain 724, are indistinguishable as test antigens but P-10 is a better immunizing antigen, and (c) that both P-5 and P-10 are inferior in these respects to P-3 from the G. W. strain.

The γ -globulin fractions obtained from the various lots of pooled antisera were examined serologically, by the precipitin and agglutinin tests. Table V contains these results together with comparable data on the pooled antisera from which the γ -globulins were derived. Studies were made also with the albumin and other globulin fractions of the antisera. The albumin fraction did not contain any demonstrable antibodies toward the polysaccharides. The ammonium sulfate fractionation,

toward P-3.F as developed by rabbits No. 28, 36, 37 and 38. Those designated P-3.F (b) were the same solutions which had stood several weeks in the refrigerator and in addition had become heated accidentally to 25 to 30° for 12 hours. The solutions designated P-3.F (c) were carefully kept antisera and γ -globulin fractions from rabbits No. 73, 74, 75 and 76.

It will be noted from this table that in the reactions with test antigen P-3.F, the 3 types of antisera were equivalent in potency as the end point of the precipitin reaction was obtained when the antisera were diluted to contain 0.1 mgm. of total protein per 0.2 cc. The γ -globulin fraction P-3.F (a), however, was 10 times more potent in antibody content than the antiserum from which it was obtained. The period of standing appeared

TABLE V: PRECIPITIN AND AGGLUTININ REACTIONS WITH POOLED ANTISERA AND γ -GLOBULIN FRACTIONS
Antisera (As.) and γ -Globulin Fractions (γ -gl.)

Test Antigens	P-3.F (a)		P-3.F (b)		P-3.F (c)		P-3.N		P-5		P-5 ach		P-10		P-10 ach	
	As.	γ -gl.	As.	γ -gl.	As.	γ -gl.	As.	γ -gl.	As.	γ -gl.	As.	γ -gl.	As.	γ -gl.	As.	γ -gl.
P-3.F	0.1	0.01	0.1	0.03— 0.1	0.1	0.1	0.4	0.1	—	—	—	—	—	—	—	—
P-5	—	—	—	—	—	—	—	—	12	—	6	—	0.75	—	0.75	0.1
P-10	—	—	—	—	—	—	—	—	—	—	6	—	1	—	0.75	0.2
Strain 724 orgs.	—	—	—	—	1	—	1.5	0.7	1.5	0.05	0.05	—	—	—	0.05	0.1

Antisera P-3.F (a) from rabbits No. 28, 36, 37, 38 (when freshly prepared)

Antisera P-3.F (b) from rabbits No. 28, 36, 37, 38 (after long standing)

Antisera P-3.F (c) from rabbits No. 73, 74, 75, 76 (when freshly prepared)

The results in this table are expressed in terms of the number of mgm. of total protein contained in the greatest dilutions both of antisera (As.) and γ -globulin (γ -gl.) producing the characteristic end point with the test antigens.

however, did not concentrate all the antibodies in the γ -globulin fraction. The α - and β -globulin fractions were found to contain serologically reactive components, to a lesser extent, however, than the γ -globulin fraction. This is not surprising since it has been demonstrated by other investigators (3, 4) that the more efficient ethanol method of fractionation is required for more satisfactory isolation of the antibody-containing fraction.

In the precipitin tests, 50 γ of polysaccharide in 0.2 cc. of saline was used. Approximately a half-billion organisms in 0.5 cc. of saline were used in the agglutination tests. The results in Table V are expressed in terms of the number of mgm. of protein contained in the greatest dilutions of (a) antisera and (b) γ -globulin producing a characteristic \pm end point with the test antigens. Since the γ -globulin fractions were to be used for studies of the influence of passive immunization against the toxic reactions of the polysaccharides, the importance of assays of the potencies of the γ -globulin fractions is obvious. The designation P-3.F (a) represents both the antisera and a freshly dialyzed solution of the γ -globulin fraction of antisera

to have no influence on the antiserum but it did decrease the potency of the γ -globulin fraction, P-3.F (b), 3-fold at first and then 10-fold. The γ -globulin fractions P-3.F (c) and P-3.N were never so potent as P-3.F (a). It was found also that the most satisfactory 2 plus serological reaction was obtained with P-3.F (a) at a level of antiserum of 1.5 mgm. of protein and at a level of 0.4 mgm. of γ -globulin fraction. No precipitin reactions were observed with the γ -globulin fractions obtained from antisera toward P-5 or P-5 ach, although weak reactions were obtained with the antisera. Both the antisera toward P-10 and P-10 ach and the γ -globulin fractions toward P-10 ach gave moderately strong reactions, however, with both the P-5 and P-10 polysaccharides. Although the potency of this γ -globulin was comparable to those of P-3.F (b) and (c), it was only 1/10 that of P-3.F (a). The agglutination tests, on the other hand, demonstrated differences existing between both antisera and γ -globulins toward P-5 and those toward P-5 ach, thus confirming the results obtained by the complement-fixation technic.

It is apparent that of all the lots of polysacchar-

ide studied, P-5 was the least antigenic and therefore, from the immunological standpoint, is the most satisfactory for clinical work. Of the P-3 preparations, the fractionated products P-3.Rb and P-3.Rb-3 were the least antigenic.

Routine serological investigations have been conducted on samples of blood obtained from 30 patients with various types of malignancy before, and at several intervals after, they had received injections of the P-5 preparation. These studies were made to determine whether the patients developed antibodies toward P-5 and, if so, to attempt to ascertain what role the antibodies played in interfering with the effectiveness of subsequent injections of P-5. Although it has been demonstrated that antibodies are developed in animals and also in human beings as a result of injections of these polysaccharides, it is not known yet that the rapid decrease in the activity of subsequent injections of polysaccharides both with regard to tumor-necrotization and toxicity is caused by a true immunological mechanism or by the development of a non-immunological tolerance. Experiments are in progress to determine whether both mechanisms or only an immunological one are responsible for the decrease in response.

Complete results of the serological studies on patients together with other information on the patient's reactions to P-5 will be published at a later date. It may be mentioned, however, that some of the patients developed antibodies as shown by the precipitin, agglutinin and complement-fixation technics. It was of interest to find that of 6 patients with Hodgkin's disease not one developed antibodies following the first series of injections. Several other patients with a variety of tumors also did not exhibit antibody formation when injected with the same or a greater amount of P-5. On the other hand, 13 patients with carcinoma, lymphosarcoma, fibrosarcoma and a variety of other cancerous conditions did develop antibodies toward P-5.

LETHAL ACTIVITY

Although it was necessary to use a miscellaneous assortment of mice for the initial toxicity experiments and in spite of the fact that the responses were not studied completely enough to decide in all cases on the 70 to 90 per cent mortality levels, certain trends may be seen. Table VI gives the results of these experiments in terms of the percentage of deaths in 24 and in 96 hours after the injection of the polysaccharide. It is obvious that P-3.Rb was the least lethal of the preparations

since the mortality at a 1.6 and a 2 mgm. dose was very low. The next was P-3.Ra which caused a 60 to 70 per cent mortality at the 2 mgm. level. The most lethal preparations were the original ones from the G.W. strain, P-3.O, P-3.N, P-3.S and P-3.F, which exhibited a 60 to 80 per cent mortality rate with a dose of approximately 400 to 500 γ of polysaccharide. Of these four, P-3.F was the most toxic.

The preparations P-5 and P-10 from the 724 strain and the fractions P-3.Rb-3 and P-3.Rb-4 from the G.W. strain appeared to be intermediate requiring about 800 to 1,000 γ to give a 60 to 80 per cent mortality. Comparison of the P-5 preparation with a sample of P-5 which had been intentionally frozen (P-5.F) indicated that freezing increased the toxicity.

Certain differences in the response of the various strains of mice can be noted from the table. In general, the young A strain mice, which incidentally at 2 months of age were larger than those of the other strains, tolerated larger doses of polysaccharide.

As already mentioned, certain of the preparations were found to be lethal to rabbits when a 500- γ dose was given intramuscularly. Preparations P-3.F, P-3.S and P-3.Rb-4 caused a 50 per cent mortality in rabbits at this level. Consideration of the dosage per unit body weight shows that the polysaccharides have a much greater lethal activity for the rabbit than for the mouse. Other studies (1, 15) confirm this observation.

STUDIES OF TUMOR-NECROTIZATION

The general course of cellular response induced in sarcoma 37 by *S. marcescens* polysaccharide has been described elsewhere by Diller (5, 6). The sample of polysaccharide employed in that study was the P-3 type from which several of the polysaccharides considered in the present paper were derived. In order to compare the tumor-necrotizing potencies of the various samples, a 10- or 20- γ quantity of polysaccharide was used. The 10- γ dose is the amount of P-3 ordinarily required to produce characteristic cytological damage to the tumor and complete regression of week-old tumor implants in at least one-fourth of the mice treated. No previously undescribed types of nuclear or cellular change were encountered with these preparations. Certain differences in rate of reaction, degree and amount of tumor destruction, and duration of mitotic inhibition were observed as a result of the action of various preparations of the polysaccharides.

P-3.F and *P-3.S* caused drastic degenerative responses in the tumors as well as a high percentage of deaths. In our experience, the mortality is invariably higher in tumor-bearing than in normal mice at comparable dosage levels (compare Tables VI and VII). Furthermore, these samples produced the greatest percentage of complete tumor regression secured in the P-3 series. With both samples,

TABLE VI: MORTALITY DUE TO POLYSACCHARIDE IN NORMAL MICE

No. mice	Type of mouse	Type of polys.	Amt. in γ	Percentage of deaths in 24 hrs.	Percentage of deaths in 96 hrs.
20	A	P-3.O	400	40	60
10	A		500	60	60
10	mx		500	80	80
10	mx	P-3.N	200	20	20
10	mx		400	50	60
10	A		400	30	40
10	mx	P-3.S	100	30	60
20	mx		200	45	50
10	A		400	10	30
10	mx	P-3.F	200	30	60
10	CAF		200	30	60
10	CAF		300	50	50
10	mx		400	40	60
20	A		400	40	70
50	CAF		400	82	92
10	A		500	90	90
20	CAF		500	90	95
10	mx	P-3.Ra	1000	40	40
10	mx		2000	60	70
10	A		2000	60	70
10	mx	P-3.Rb	1600	10	20
10	mx		2000	0	0
20	mx	P-3.Rb-3	500	50	55
20	mx	P-3.Rb-4	500	25	35
20	A	P-5	400	5	25
10	A		500	0	0
10	A		625	20	40
20	Sw		750	57	62
10	A		800	40	60
10	A		1000	50	50
10	CAF		1000	100	100
20	Sw		1000	95	100
10	A		1250	50	60
25	Sw	P-5 (a)*	875	32	32
20	A	P-5.F	400	10	30
10	A		500	10	30
10	A		625	50	70
10	A		800	40	50
20	Sw	P-10	1000	80	85
45	A		1000	84	92

* P-5 (a) was a separate vial of P-5 of lower toxicity.

spotty hemorrhage was observed in the tumors at 6 hours post-treatment. Full hemorrhage in the degree previously reported for P-3 at 6 hours was not attained until some time later. Nevertheless, nuclear damage at 6 hours was pronounced and involved the production of swollen nuclei of aberrant contour, nuclear fragmentation, and attenuated chromatin filaments. At 24 hours, cell division had ceased, and was not resumed until the fourth day following treatment.

The samples *P-3.O* and *P-3.N* produced severe macroscopic hemorrhage in tumors, followed on the second or third day by formation of dark scabs and necrotic centers of sandy texture. In the early hours following polysaccharide administration, cellular destruction was slight and few chromatin filaments were observed. The reaction appeared to progress more slowly in all stages and morphological changes were less drastic. The sample *P-3.O* produced noticeable changes in the staining capacity of undestroyed cells at 24 hours. Orcein staining was murky and diffused, as though there had been alteration of nuclear substance without actual disintegration of structure. Few of the tumors regressed completely and division was re-established in healthy tissue on the third day post-injection.

Fraction *P-3.Ra*, which caused the death of few treated animals, produced various degrees of hemorrhage in the tumors. Nevertheless, typical filamentous degeneration, plus crescent-shaped and otherwise degenerative nuclei, appeared at 6 hours in large areas of some tumors, although healthy resting cells were found in abundance in those tumors in which hemorrhage was only slight. At 24 hours, one of every 3 tumors was covered with dried blood overlying white and opaque necrotic tissue. Division was resumed on the third day in the remainder of the tumors.

The preparation *P-3.Rb* was considerably less toxic to tumor-bearing mice than were the other preparations; in this series, no animals died as a result of the treatment. No gross hemorrhage was produced in the tumors at 6 hours, but microscopic examination 24 hours after injection revealed considerable areas of degeneration in some tumors. Nuclei were fragmentary, filamentous, and in some cases were absent. Resting cells and even some dividing ones were found dispersed in the necrotic tissue. No tumors responded completely to treatment, but were able to re-establish themselves after the usual 3-day interval.

P-3.Rb-3 and *P-3.Rb-4*, on the other hand, produced decided hemorrhage in tumors at 6 hours, comparable to that ordinarily induced by P-3. At that time, the tumor tissue was partially coagulated, and the nuclei were shrunken, degenerative and hazily stained. At 24 hours, several of the tumors seemed diminished in size and extreme cellular damage was encountered in these cases. Nuclear membranes were crenated and the central portion of the nucleus was often vacuolated. Some nuclei were folded and twisted, while others were broken into minute fragments. The tissue was very resistant to smearing and appeared coagulated. No

dividing cells were found, either at 24 hours or at 3 days.

P-5 and *P-10*.—The results of studies of the lethal action of these preparations in 3-month old Swiss mice bearing sarcoma 37 transplants 10 to 15 mm. in diameter are recorded in Table VII. Prep-

TABLE VII: LETHAL ACTION OF POLYSACCHARIDES IN SWISS MICE BEARING SARCOMA 37

No. of mice	Type of polysacc.	Amt. in γ	Percentage of deaths in	
			24 hrs.	96 hrs.
27	P-3.F	10	26	33
45		50	60	60
38	P-5	20	3	21
20	P-5a	200	35	40
45	P-10	10	15	27
45		20	13	20
24		50	8	21
20		200	20	30
37		250	65	70

aration P-3.F was found to be more toxic than either P-5 or P-10. It may be seen readily by comparison with Table VI that tumor-bearing mice are more susceptible to the lethal action of the polysaccharides than are normal mice. Tumors responded rapidly to both P-5 and P-10 and displayed pronounced hemorrhage and drastic nuclear degeneration at the 20- γ dose. Although the regression rate was determined, the results are not considered satisfactory because of the high rate of spontaneous regression in the control mice. About 55 per cent regression was observed in the 128 mice injected with 10 or 20 γ of P-5 or P-10 compared with about 20 per cent spontaneous regression in a control series of 60 mice.

Comparisons of the tumor-necrotizing activities of the various preparations as judged by the cytological responses of the tumors to the polysaccharides are included in Table VIII.

GROSS PATHOLOGICAL EXAMINATION (E. T. NISHIMURA)

The gross pathological findings in normal mice that died within 24 to 96 hours after the injection of massive doses of the P-3 and P-5 type polysaccharides were based on necropsies of 79 animals which were examined shortly after death. The most striking observation was that of pulmonary congestion which was found in 67 mice. This varied in appearance from bright pink to grossly hemorrhagic lungs which often were edematous. Of these 67 mice, about 1/3 had right ventricular dilatation of the heart. The livers in about 2/3 of the entire series were pale and mottled and a significant number of these showed some tendency toward enlarge-

ment. The small intestine in several instances showed congestion of the mucosa particularly in the upper segments. Pale and mottled kidneys were encountered in some cases but no appreciable alteration in size was noted. About half of the adrenals examined appeared darker than normal (reddish brown). Splenomegaly occurred in a considerable number of mice. The lymph follicles were often quite prominent on the cut surface and a moderate degree of congestion was present. A generalized enlargement of lymph nodes was seen in half of the animals and hyperemia of the surrounding tissues was seen commonly in the inguinal groups. Peyer's patches often were prominent. The thymus glands occasionally showed evidence of congestion but rarely were enlarged.

The results of histopathological studies on normal tissues as well as on tumors in mice and rabbits treated with polysaccharides will be reported at a later date by Dr. A. J. Donnelly of this Institute.

EFFECT OF POLYSACCHARIDE ON CARCINOMA

Since we had available from another project,⁵ 14 rabbits bearing carcinomas produced by the application of 9,10-dimethyl-1,2-benzanthracene to the ears, tests were made to determine whether the polysaccharides P-3.F, P-5 and P-10 would have any action on these tumors. As expected from other observations (1, 15) the polysaccharides exerted very little tumor-necrotizing action on these carcinomas except when given in amounts which were either lethal or nearly lethal. On the other hand, some rabbits died as a result of the injection of polysaccharides without showing any hemorrhage or necrosis of the tumors. As low an amount as 50 γ of P-5 given intravenously killed several rabbits; their tumors showed considerable hemorrhage and necrosis. Repeated intramuscular injections of 500 γ of P-5 for four successive days caused a slight amount of hemorrhage in the carcinomas of the two rabbits so treated. Thirty days later, these rabbits were injected intravenously with 500 γ of P-5. This amount was not lethal; considerable damage was produced in the tumors as determined by biopsy before and after treatment and by cytological studies. Reference already has been made to the high lethal action of the polysaccharides in rabbits.

⁵"Immunological Properties of Conjugates Prepared from Proteins and Isocyanates of Polynuclear Aromatic Hydrocarbons." A review presented at the Fourth International Cancer Research Congress, St. Louis, Sept. 2 to 7, 1947.

SUMMARIZED COMPARISON OF PROPERTIES

A comparison of the various preparations of polysaccharides is given in Table VIII. The designation of unity represents the greatest toxicity and greatest antigenicity. Numerical fractions arbitrarily have been assigned to represent the relative

TABLE VIII: SUMMARIZED COMPARISON OF POLYSACCHARIDES

Polysacc.	Relative antigenicity	Relative lethal action in normal mice	Relative tumor necrotization (cytological response)
P-3.O	1	1	slight at 10 γ
P-3.N	1	1	slight at 10 γ
P-3.S	1	1	moderate at 10 γ
P-3.F	1	1	pronounced at 10 γ
P-3.Ra	1	1/5	moderate at 10 γ
P-3.Rb	1/10	1/10	moderate at 10 γ
P-3.Rb-3	1/3	1/2	moderate at 10 γ
P-3.Rb-4	1	1/3	moderate at 10 γ
P-5	1/20	1/2	moderate at 20 γ
P-10	1/5	1/2	moderate at 20 γ

potencies of products of lower activity. Estimates of tumor-necrotizing activity, which are far less accurate than the other comparisons, were based on the cytological response of the tumors to 10 or 20 γ of polysaccharide. In general, it was found that the original preparations, P-3.O, P-3.N and P-3.S, ran parallel in the toxic and antigenic responses. Storage of the polysaccharide reduced the tumor-necrotizing ability, however, as shown by the actions of P-3.O and P-3.N. Freezing of the solution of polysaccharide enhanced the tumor-necrotizing action considerably and also increased the toxic and antigenic properties slightly. Fractionation of the solution of frozen polysaccharide gave products (P-3.Ra, P-3.Rb, P-3.Rb-3 and P-3.Rb-4) in which the properties did not run parallel. It is not certain that the simple processes of fractionation caused a true separation of components of the original preparation. It is possible that the method merely separated products which may have been formed in the original preparation as a result of freezing and of possible contamination with molds and bacteria.⁶

Of the products obtained in this manner, P-3.Rb was the best because of its relatively low antigenicity and toxicity and moderate tumor-necrotizing ability. It seems reasonable to conclude from these observations that it should be possible to obtain preparations with a much better combination of properties than that in the original preparation of polysaccharide from the G.W. strain.

The recently acquired preparations, P-5 and P-10, from the 724 strain, in general exhibited more

satisfactory properties than those from the other strain. Of the two, P-5 was the better because of its weaker antigenic properties. The toxicities of the two preparations were approximately equivalent in normal mice. Moderate tumor-necrotizing activities were displayed by both P-5 and P-10. From our studies, it would seem that P-10 is far less toxic in tumor-bearing mice than the P-3 type polysaccharide.

SUMMARY

Comparisons have been made of the immunological, lethal and tumor-necrotizing activities of purified polysaccharides obtained from culture filtrates of the G.W. and 724 strains of *Serratia marcescens*. Studies of these properties were made also on products of fractionation of the polysaccharide from the G.W. strain. Considerable variations in these properties were found between the 2 strains and particularly among the isolated fractions. The P-3 type polysaccharides obtained from the G.W. strain were found to be about twice as toxic both to normal mice and mice bearing sarcoma 37 and several times as antigenic in rabbits as the P-5 and P-10 polysaccharides from the 724 strain. The tumor-necrotizing activities of the various polysaccharides seemed to show less variation. The polysaccharides from the 2 strains were found not to be related serologically. Simple fractionation of a preparation of polysaccharide from the G.W. strain led to the isolation of some products which had only weak antigenicities and toxicities but moderate tumor-necrotizing activities. Storage of the solutions of the polysaccharide at room temperature for a period of several years caused a definite decrease in tumor-necrotizing ability but caused no significant alteration either in toxicity or antigenicity. Freezing of a solution of the polysaccharide seemed to enhance the tumor-necrotizing activity without affecting the other properties to any considerable extent. Because of their weaker antigenicities and toxicities both in normal and tumor-bearing mice, the P-5 and P-10 polysaccharides from the 724 strain have definite advantages over the P-3 types from the G.W. strain.

REFERENCES

1. BECK, L. V., and FISHER, M. Physiological Studies on Tumor-Inhibiting Agents. II. Effect on Rectal Temperatures in Normal Rabbits of the *Serratia marcescens* Tumor-Necrotizing Polysaccharide of Shear. *Cancer Research*, 6:410-420. 1946.
2. CHASE, JEANNE H., WHITE, A., and DOUGHERTY, T. F. The Enhancement of Circulating Antibody Concentration by Adrenal Cortical Hormones. *J. Immunol.* 52:101-112. 1946.

⁶Studies of the action of such contaminants are being contemplated at the National Cancer Institute.

3. COHN, E. J., STRONG, L. E., HUGHES, W. L., JR., MULFORD, D. J., ASHWORTH, J. N., MELIN, M., and TAYLOR, H. L. Preparation and Properties of Serum and Plasma Proteins. IV. A System for the Separation into Fractions of the Protein and Lipoprotein Components of Biological Tissues and Fluids. *J. Am. Chem. Soc.*, **68**:459-475. 1946.
4. DEUTSCH, H. F., GOSTING, L. J., ALBERTY, R. A., and WILLIAMS, J. W. Biophysical Studies of Blood Plasma Proteins. III. Recovery of γ -Globulin from Human Blood Protein Mixtures. *J. Biol. Chem.*, **164**:109-118. 1946.
5. DILLER, IRENE C. Approaches to Tumor Chemotherapy. A.A.A.S. Lancaster, Pa.: The Science Press Printing Company. 1947, p. 260.
6. DILLER, IRENE C. Degenerative Changes Induced in Tumor Cells by *Serratia marcescens* Polysaccharide. *Cancer Research*, **7**: 605-626. 1947.
7. FRANKE, F. E. Attempts to Produce Anaphylaxis in Guinea Pigs with a Polysaccharide from *Serratia marcescens* (*Bacillus prodigiosus*). *J. Nat. Cancer Inst.*, **5**:173-177. 1944.
8. HARTWELL, J. L., SHEAR, M. J., and ADAMS, J. R., JR. Chemical Treatment of Tumors. VII. Nature of the Hemorrhage-Producing Fraction from *Serratia marcescens* (*Bacillus prodigiosus*) Culture Filtrate. *J. Nat. Cancer Inst.*, **4**:107-122. 1943.
9. KOLMER, J. A., and BOERNER, F. Approved Laboratory Technic. Clinical, Pathological, Bacteriological, Mycological, Parasitological, Serological, Biochemical and Histological. Third Edition, New York and London: Appleton-Century Company. 1941, p. 616.
10. MCMECKIN, T. L. Serum Albumin. I. The Preparation and Properties of Crystalline Horse Serum Albumin of Constant Solubility. *J. Am. Chem. Soc.*, **61**:2884-2887. 1939.
11. SHWARTZMAN, G. Phenomenon of Local Skin Reactivity to *Serratia marcescens* (*B. prodigiosus*). Immunological Relationships between *Serratia marcescens* Culture Filtrates and Shear Polysaccharide. *Cancer Research*, **4**:191-196. 1944.
12. SHEAR, M. J., and PERRAULT, A. Chemical Treatment of Tumors. IX. Reactions of Mice with Primary Subcutaneous Tumors to Injections of a Hemorrhage-Producing Bacterial Polysaccharide. *J. Nat. Cancer Inst.*, **4**:461-476. 1944.
13. SHEAR, M. J., PERRAULT, A., and ADAMS, J. R., JR. Chemical Treatment of Tumors. VI. Method Employed in Determining the Potency of Hemorrhage-Producing Bacterial Preparations. *J. Nat. Cancer Inst.*, **4**:99-105. 1943.
14. SHEAR, M. J., and TURNER, F. C. Chemical Treatment of Tumors. V. Isolation of the Hemorrhage-Producing Fraction from *Serratia marcescens* (*Bacillus prodigiosus*) Culture Filtrate. *J. Nat. Cancer Inst.*, **4**:81-97. 1943.
15. WESTFALL, B. B., and DUNN, THELMA B. Blood Uric Acid and Proteose, Body Temperature, and Glomerular Clearance of Rabbits Implanted with the Brown-Pearce Carcinoma and Treated with the Polysaccharide from *Serratia marcescens*. *J. Nat. Cancer Inst.*, **7**:115-121. 1946.

The Influence of Antibody-Containing Fractions on the Lethal and Tumor-Necrotizing Actions of Polysaccharides from *Serratia marcescens* (*Bacillus prodigiosus*)*

Hugh J. Creech, Ph.D., Mary Alice Hamilton, Ph.D., Edwin T. Nishimura, M.D., and Reed F. Hankwitz, Jr., B.S.

(From the Lankenau Hospital Research Institute and the Institute for Cancer Research, Philadelphia 30, Pa.)

(Received for publication December 10, 1947)

Because of certain undesirable reactions, such as elevation in temperature and drop in blood pressure, observed in patients receiving tumor-necrotizing polysaccharides, it is important to investigate procedures which have potentialities for reducing the toxicity of the polysaccharides without interfering with the tumor-necrotizing action.

It seemed advisable, therefore, to ascertain whether passive immunization with rabbit antisera toward the polysaccharides from *Serratia marcescens* would decrease either the lethal or the tumor-necrotizing actions (or both) of these polysaccharides in mice. Schwartzman (5) found that certain mixtures of *Bacillus typhosus* filtrate, and antityphoid horse serum upon injection into mice with sarcoma 180 protected the mice against early death without diminishing the extent of hemorrhage and necrosis of the tumors. The preparation of antisera toward the polysaccharides has been discussed in the preceding paper (2). In the first series of tests to be described, normal mice were immunized passively with the γ -globulin fraction from antisera toward the polysaccharides prior to the injection of lethal doses of the polysaccharides. In the second series of experiments, mice bearing sarcoma 37 were immunized passively with these γ -globulin fractions and then injected with relatively large tumor-necrotizing doses of polysaccharides.

EXPERIMENTAL

Three polysaccharides, P-3.F, P-5 and P-10, which were available in adequate amounts for these studies, were assayed to determine the amounts of each needed to kill a high percentage of normal mice within 96 hours. An intraperitoneal injection of 400 γ of P-3.F, 1,000 γ of P-10, and 750, 875 and 1,000 γ of P-5 were employed

for this purpose. The quantities of γ -globulin fraction used in the experiments were based on the results of the serological tests reported in the preceding paper (2). After isolation from the antisera, the γ -globulin fractions were stored in the refrigerator under 2 M ammonium sulfate solution. When required for a series of tests, portions of the globulin suspension were dissolved in water and dialyzed against distilled water and physiologic saline; micro-Kjeldahl analyses were made on the ammonium sulfate-free solutions of γ -globulin and these solutions were diluted with physiologic saline so as to contain the required amount of protein per selected volume. Merthiolate (Lilly) was added to give a final concentration of 1 : 10,000.

In the case of antisera toward P-3.F, 3 or 6 mgm. of γ -globulin fraction in 0.25 cc. or in 0.5 cc. of physiologic saline was used to combat the lethal action in a normal mouse of 400 γ of P-3.F in 0.25 cc. of physiologic saline. Because of the lower antibody content of the antisera toward P-5 and P-10, greater amounts of γ -globulin were employed. From preliminary trials, it seemed that the intraperitoneal injection of the globulin fraction 3 hours before the administration of the polysaccharide was the most effective timing for protection. Except for preliminary studies, the results of which are not recorded in this paper, the mice were about 2 months of age and were "A" strain, or a "Swiss" strain obtained commercially, or mice obtained by the mixed breeding of descendants of a cross between the A and C strains. In general, several series of 10 mice (either all males, or 5 males and 5 females) were used in the experiments in order to permit adequate comparisons among the control mice and those receiving the various γ -globulin fractions. No significant differences have been noted between the responses of normal male and female mice of any one strain at any given polysaccharide level.

* Presented in part at the 38th Annual Meeting of the American Association for Cancer Research, Chicago, May 16 and 17, 1947.

In the experiments designed to determine the influence of passive immunization on the lethal and tumor-necrotizing actions of the polysaccharide P-3.F, transplants of sarcoma 37 were made subcutaneously into the dorsum of Swiss mice 2 months of age. Usually 10 days after transplantation, the tumors had reached a size of 15 to 20 mm. in diameter and had become well established in their

to determine the extent of regression of the tumors in these strains of mice.

RESULTS

An outline of the experimental conditions and the results of the studies of the effectiveness of γ -globulin fractions from various antisera in protecting normal mice against the lethal action of

TABLE I: PASSIVE IMMUNIZATION VS. LETHAL ACTION OF P-3.F POLYSACCHARIDE IN NORMAL MICE

Line	γ -globulin	Amt. mgm.	Exper. cond.	Polys.	Amt. γ	No. mice	Type	Per cent deaths	
								In 24 hrs.	In 96 hrs.
1	—	0	—	P-3.F	400	50	CAF	82	92
2	γ -glob.a/P-3.F	3, 6	—	—	0	10	"	0	0
3	" /normal	3, 6	a	P-3.F	400	19	"	70	90
4	" a/P-3.F	3, 6	a	"	400	60	"	18	25
5	" b/P-3.F	1, 3, 6	a	"	400	70	"	39	53
6	" a/P-3.F	3, 6	b	"	400	30	"	23	42
7	" c/P-3.F	3, 6	a	"	400	40	"	65	95
8	" a/P-3.F	3	c	"	400	10	"	70	70
9	" /P-3.N	3, 6	a	"	400	30	"	30	33
10	" /P-3.Rb	2	a	"	400	19	"	16	26
11	" /P-5	4, 8	a	"	400	30	"	77	87
12	—	0	—	P-3.M	800	45	A	85	92
13	γ -glob.m/P-3	12	a	"	800	40	"	13	35
14	" 2/P-10	14	a	"	800	15	"	27	53
15	antiserum /P-5	1cc.	a	"	800	10	"	50	100
16	" 2/P-10	1cc.	a	"	800	40	"	17	70
17	" 2/P-10	1cc.	—	—	—	10	"	0	0

γ -glob.a/P-3.F was from antisera of rabbits No. 28, 36, 37, 38 (when freshly prepared)

γ -glob.b/P-3.F was from antisera of rabbits No. 28, 36, 37, 38 (after long standing)

γ -glob.c/P-3.F was from antisera of rabbits No. 73, 74, 75, 76

γ -glob.m/P-3 was a mixture of γ -globulin fractions from antisera toward P-3.F, P-3.S and P-3.O.

γ -glob.2/P-10 was from antisera of rabbits No. 97 to 103

P-3.M was a mixture of polysaccharides P-3.F, P-3.N and P-3.O.

Exper. cond. (a) γ -glob. given 3 hours before polysaccharide

(b) Simultaneous injection or as a mixture

(c) γ -glob. given 3 hours after polysaccharide

growth. Such tumor-bearing mice were considered suitable for our studies. Injection of the γ -globulin fraction was made intraperitoneally 3 hours before the polysaccharide was injected via the same route. Five times the usual amount of polysaccharide needed for demonstration of tumor-necrotizing activity by the cytological method of Diller was employed in this work because it had been found that the γ -globulin fractions protected normal mice against the lethal action of the polysaccharide. Several mice from each experimental series were sacrificed at 6 and 24 hours, and at 3 days, for microscopic examination of the tumors. The mice which survived the polysaccharide treatment were killed on the 21st day after the polysaccharide was administered. Autopsies were performed either to determine the size of the tumor or to confirm regression of the tumor. Since sarcoma 37 sometimes regresses spontaneously, mice not injected with polysaccharide were used as controls

the P-3.F polysaccharide are given in Table I. In order to limit the size of the table, the results of comparable series of tests have been combined. Thus, a series of 5 tests with 10 mice each were conducted at the initial, middle and final periods of the experiment to obtain the information on line 1 of the table. The results of the tests are expressed as per cent deaths within 24 and within 96 hours after the polysaccharide was injected. It was found that the majority of the deaths due to polysaccharide occurred within 24 hours, and that after 96 hours the number of deaths was insignificant.

In every case, whether or not γ -globulin was administered, the mice became prostrated about 3 hours after the polysaccharide was injected. The passively immunized mice, however, appeared to be less sick and to recover more rapidly than those which were not given the antibody-containing fraction. Of those mice that died as a result of polysac-

charide action, it was noted that the passively immunized mice on the average survived several hours longer than the control mice.

From line 1 of Table I, it may be seen that 400 γ of P-3.F was sufficient to kill 82 per cent of the CAF mice within 24 hours, and 92 per cent of the 50 mice within 96 hours. As was to be expected, the injection of the γ -globulin fraction (γ -glob. a/P-3.F) alone caused no deaths in the mice (line 2). Injection of either 3 mgm. or 6 mgm. of normal γ -globulin (isolated from the serum of untreated rabbits), 3 hours prior to the injection of 400 γ of P-3.F, had no significant influence on the mortality (line 3).

In the experiments listed in line 4, 3 mgm. and 6 mgm. of γ -globulin of high antibody titer (see Table V of the preceding paper) obtained from antisera toward P-3.F were administered to 60 mice 3 hours prior to the injection of the polysaccharide P-3.F. This preparation afforded pronounced protection against the lethal action of the polysaccharide, lowering the mortality from 82 to 18 per cent at the 24-hour period and from 92 to 25 per cent at the 96-hour period. Among these experiments, there was one with ten mice in which no deaths occurred.

In the next experiment (line 5) there was employed a dialyzed solution of γ -globulin toward P-3.F which, because of circumstances beyond our control, had undergone about a five-fold reduction in antibody potency as shown by serological tests. This preparation, designated γ -glob.b/P-3.F, showed about half the effectiveness of the original preparation (γ -glob.a/P-3.F) in protecting the mice against the lethal action of P-3.F. For some reason, the fraction γ -glob.c/P-3.F, obtained from antisera formed in the second series of rabbits immunized with P-3.F, had a much lower antibody content than γ -glob.a/P-3.F in spite of the serological equivalence of the 2 sets of pooled antisera. It will be observed that this particular fraction (γ -glob.c/P-3.F) had no significant effect, at either the 3 or 6 mgm. level, in reducing the lethal action of the polysaccharide (line 7).

Simultaneous injection of P-3.F and γ -glob.a/P-3.F or a single injection of a mixture of the two components also afforded good protection (line 6). Since the polysaccharide seemed to exhibit pronounced action from 3 to 6 hours after injection, a test was made to see whether or not the injection of 3 mgm. of γ -glob.a/P-3.F 3 hours after the administration of P-3.F would have any beneficial effect. From line 8, it would seem that there was little, if any, protection although the number of

mice used may not allow strict comparison with the other tests. It did not appear, however, to be a promising lead and so it was not pursued further.

It is interesting to note that the γ -globulin fractions from antisera toward P-3.N and P-3.Rb (lines 9 and 10) also protected the mice against the lethal action of P-3.F. In the case of γ -glob./P-3.N, this is not surprising because of the serological relationships of these two polysaccharides and their antisera. It is of considerable significance, however, that 2 mgm. of γ -glob./P-3.Rb, which showed only extremely weak serological reactions with any of the polysaccharides, afforded strong protection against the lethal action of 400 γ of P-3.F. This would indicate that the absence of antibodies of the complement-fixing and precipitin type does not mean necessarily that protective antibodies are absent. Such an observation is not unique, of course, because similar phenomena have been noted on occasion in other immunological investigations (1, 3).

The γ -globulin toward P-5 which showed no serological reactions with the P-3 type polysaccharides also exhibited no protective effect against P-3.F when given at the 4 and 8 mgm. levels (line 11).

Since it seemed desirable to investigate the protective aspects further and since the supply of P-3.F was limited, certain P-3 type polysaccharides were mixed so that enough uniform material would be available for a series of controlled tests. This preparation was designated P-3.M and consisted of a mixture of P-3.F, P-3.N and P-3.O. When assayed for its lethal activity in A strain mice 2 to 3 months of age, it was found that an 800- γ intraperitoneal injection of P-3.M caused the death of 85 per cent of the mice within 24 hours and 92 per cent within 96 hours (line 12). The administration of 12 mgm. of a mixture of γ -globulins, designated γ -glob.m/P-3, from antisera toward P-3.F, P-3.S and P-3.O, prior to the injection of 800 γ of P-3.M afforded definite protection against the lethal action of the polysaccharide mixture (line 13). It will be noted that this ratio of γ -globulin to polysaccharide (12 : 800) is equivalent to that often used with P-3.F.

When the γ -globulin fraction (γ -glob.2/P-10), from antisera elicited by the injection of the polysaccharide P-10 into a second series of rabbits, was injected into mice before the injection of P-3.M, it was found to have but little influence on the lethal action of the polysaccharide other than a delaying action (line 14). Similarly the use of 1 cc. of antisera (containing about 60 mgm. of protein per cc.) toward either P-5 or P-10, as shown in lines 15 and 16, had no appreciable effect on the lethal action

of P-3.M. The antiserum toward P-10 when administered alone did not kill any mice (line 17).

Thus, neither the antisera toward P-5 nor the γ -globulin fraction of these antisera, which contained an antibody content which was both low in amount and serologically unreactive toward the P-3 type polysaccharide, had any protective action against the P-3.M polysaccharide. On the other hand, the antisera toward P-10 and the γ -globulin fraction of these antisera, which contained a moderate content of antibody serologically reactive toward P-5 and P-10 but not toward the P-3 types, exerted a delaying action at least, on the lethal action of the P-3.M polysaccharide. This antiserum and its γ -globulin fraction may have decreased to some extent the lethal action of P-3.M, but from evidence presented in Table II, this effect probably is not significant.

The next series of studies involved the use of the P-5 and P-10 polysaccharides. All the γ -globulin fractions were administered 3 hours before the P-5 or P-10 in these studies. The mice for the first experiment were obtained by the mixed breeding of descendants of a cross between C and A strain mice. From line 1, Table II, it is seen that 1,000

γ of P-5 killed a high percentage of mice. The use of normal γ -globulin reduced the mortality slightly (line 2) but the reduction is not considered to be significant. A similar slight reduction was caused when γ -glob.a/P-3.F was administered before P-5. When γ -glob./P-5 at the 4, 8, and 10 mgm. levels was employed (line 4) no protection was obtained against the lethal action of P-5.

Swiss mice were used in the next series of experiments; practically all the mice were males. In this series, 750 γ of P-5 was employed to obtain a standard mortality rate (line 5). From lines 6 and 8, it will be noted that 20 mgm. of γ -glob./P-5ach or of γ -glob./P-10ach had essentially no action in protecting the mice. Similarly, the γ -globulin fraction (γ -glob.h/P-5), obtained from a patient who showed a high antibody titer by the three serological methods following a series of injections of P-5, did not show any protective action even at a 29 mgm. level (line 7).

To confirm these results, a higher dosage of P-5 was investigated. This lot of P-5 came from a separate vial which had been delayed during shipment from Dr. Shear; it was designated P-5a when the lethal potency was found to be considerably

TABLE II: PASSIVE IMMUNIZATION VERSUS LETHAL ACTION OF P-5 AND P-10 POLYSACCHARIDES IN NORMAL MICE

Line	γ -globulin	Amt. mgm.	Polys.	Amt. γ	No. mice	Type mouse	Per cent deaths	
							In 24 hrs.	In 96 hrs.
1		0	P-5	1000	10	CAF	90	100
2	γ -glob. /normal	10	"	1000	10	"	50	70
3	γ -glob.a/P-3.F	3, 6	"	1000	12	"	50	75
4	γ -glob. /P-5	4, 8, 10	"	1000	30	"	65	90
5		0	"	750	21	Sw	57	62
6	γ -glob. /P-5ach	20	"	750	20	"	35	50
7	γ -glob.h/P-5	29	"	750	22	"	45	54
8	γ -glob. /P-10ach	20	"	750	20	"	35	50
9		0	P-5a	875	25	"	32	32
10	γ -glob. /normal	20	"	875	20	"	60	80
11	" /P-5	30	"	875	10	"	20	40
12	" /P-5ach	20	"	875	10	"	30	40
13	" h/P-5	20	"	875	10	"	50	60
14	" /P-10ach	20	"	875	10	"	30	40
15		0	P-10	1000	20	"	80	85
16	γ -glob. /normal	20	"	1000	13	"	77	85
17	" /P-5	30	"	1000	10	"	50	60
18	" /P-5ach	20	"	1000	10	"	80	90
19	" h/P-5	20	"	1000	10	"	60	80
20	" /P-10ach	20	"	1000	10	"	90	90
21	" /normal	20	—	0	4	"	0	0
22	" /P-5-10	20	—	0	9	"	0	0
23		0	P-10	1000	61	A	84	92
24	γ -glob.m/P-3	12	"	1000	40	"	58	83
25	" m/P-3	24	"	1000	15	"	33	87
26	" 2/P-10	14	"	1000	14	"	14	50
27	normal serum	1cc.	"	1000	10	"	40	80
28	antiserum m/P-3	1cc.	"	1000	15	"	73	93
29	" /P-5	1cc.	"	1000	10	"	30	80
30	" 2/P-10	1cc.	"	1000	30	"	10	27

Polysaccharide P-5a was a separate lot of P-5 apparently of lower toxicity.

γ -glob.h/P-5 was obtained from a patient showing high antibody titer after a series of P-5 injections.

lower than the usual supplies of P-5. From line 9, it may be seen that 875 γ of P-5a killed only 32 per cent of the mice within 24 and 96 hours. The results in lines 11 to 14 demonstrate again that the γ -globulin fractions of antisera toward P-5 and P-10, even at the 20 mgm. level, had no influence on the lethal action of 875 γ of P-5a.

An anomalous result is recorded in line 10. In this instance, the administration of 20 mgm. of normal γ -globulin before P-5 apparently increased the mortality rate. In order to administer a 20 mgm. amount of the γ -globulins, a volume of solution greater than usual was used for the injection. Previously, the amounts of γ -globulin in the range of 1 to 10 mgm. were contained either in 0.25 or in 0.5 cc. of saline whereas the 20 mgm. amount was given in 1.0 cc. saline; the 875 γ of P-5 was contained in 0.5 cc. of saline. When 20 mgm. either of normal globulin or of γ -globulin toward P-5 or P-10 in a 1 cc. volume was given intraperitoneally followed 3 hours later by 0.5 cc. of normal saline, it was noted that none of the mice died (lines 21 and 22). Hence the volume effect alone does not explain the results of the normal γ -globulin; moreover, it does not seem probable that the injection of a total volume of 1.5 cc. instead of 1 cc. plays any significant part in the lack of protective action of γ -globulin fractions from antisera toward P-5. In fact, Rosenthal (4) found large volumes of saline to be effective against traumatic, hemorrhagic and burn shock in mice.

The polysaccharide P-10, when used at the 1,000 γ level, was found to kill a high percentage of the Swiss mice (line 15). Normal globulin, when given at the level of 20 mgm. in 1 cc. of saline, had no effect in preventing the action of P-10 (line 16); moreover, it did not increase the mortality rate. The latter finding is further evidence that the effect of a 1.5 cc. volume rather than 0.5 to 0.75 cc. was not of significance in explaining the results in line 10. There is no evidence from lines 17 to 20 that antibody fractions of antisera toward either P-5 or P-10 had any influence on the lethal action of P-10.

At this point, the investigations were continued with the use of A strain mice 2 to 3 months of age. Upon assay for lethal activity, it was found that 1,000 γ of P-10 killed a high percentage of the A strain mice (line 23). When γ -glob.m/P-3, the mixture of γ -globulin fractions of antisera toward 3 lots of P-3, was administered before the P-10 polysaccharide, it was found not to have any significant effect on the lethal action of that polysaccharide even when given at high levels (lines 24 and 25).

It has been shown previously (line 13, Table I) that this γ -globulin fraction was definitely effective in preventing the lethal action of P-3.M. From line 26 of Table II, it is seen that the γ -globulin fraction of the pooled antisera toward P-10 as produced in the second series of rabbits had a protective influence against P-10 whereas the γ -globulin fraction from antisera toward P-10 as elicited in the first series of rabbits (γ -glob./P-10ach) had shown no protective action against either P-5 or P-10 (lines 8, 14 and 20).

The effects of various antisera were studied also. From lines 27 to 29, it is seen that 1 cc. (containing about 60 mgm. of protein per cc.) of normal serum, or of antiserum toward P-3, or of antiserum toward P-5 had no significant protective action against 1,000 γ of P-10. Antiserum 2 toward P-10, however, decreased the number of deaths due to P-10 (line 30).

From the results of this series of experiments, it may be concluded that antisera toward P-3 as well as their γ -globulin fractions, while able to protect the mice effectively against the lethal action of the P-3 type polysaccharides from the G.W. strain, exert no protective action against the lethal actions of the P-5 and P-10 polysaccharides from the 724 strain. These antisera and their γ -globulin fractions had a relatively high content of antibodies which reacted serologically with the P-3 type polysaccharide but not with the polysaccharides from the 724 strain. Antisera and γ -globulin fractions toward P-5, which has poor antibody stimulating properties, have displayed no protective action against either P-5 or P-10. One lot of pooled antisera and γ -globulin fraction toward P-10 had an effect in decreasing the lethal action of P-10 whereas another lot had no action against either P-5 or P-10. In contradistinction to the findings with P-3.Rb, which showed a capability of forming highly protective but serologically weak antibodies, the P-5 polysaccharide produced antibodies which were weak both serologically and protectively.

The information in Table III is concerned with the effects of passive immunization on the lethal action of 50 γ of P-3.F in Swiss mice bearing sarcoma 37. The γ -globulin fraction of antisera toward only the P-3 type polysaccharide was used. The results are recorded as per cent survival of the mice at various intervals of time after the polysaccharide was injected. The heading "Exper. No." shows the groupings of the mice in the experiments. In all cases, it was established definitely that the tumors were growing well and were essentially uni-

form in size before the mice were used in the experiments.

It is evident immediately from Table III, that the use of the γ -globulin fractions had a pronounced influence on the lethal action of the polysaccharide in tumor-bearing mice. Considering each experimental series, the per cent survival at 1 and 4 days in the immunized mice was considerably greater than that of the non-immunized group. When averaged, the per cent survival in the latter group was about half that of the immunized group at 1 and 4 days (41 vs. 83 per cent at 4 days). Tumor-

protective studies in normal mice. Some compensation for this was made, however, by the use in these experiments of 3 times the ratio of γ -globulin to polysaccharide as that used in the protection of normal mice. An increased amount of γ -globulin probably would decrease the mortality rate even further.

The influence of passive immunization on the tumor-necrotizing action of the polysaccharide was studied by two methods. The cytological responses in the tumors removed from mice sacrificed at 6 and 24 hours, and at 3 days, were studied care-

TABLE III: PASSIVE IMMUNIZATION AGAINST THE LETHAL ACTION OF A 50- γ DOSE OF P-3.F POLYSACCHARIDE IN SWISS MICE BEARING SARCOMA 37

γ -globulin	Amt. mgm.	Exp. no.	No. mice	24 hours	Per cent survival at 4 days	10 days	21 days
Control group	0	1	10	30	30	30	20
	0	2	26	35	35	31	23
	0	3	8	63	63	63	38
γ -glob./normal	1.5	4	15	73	47	47	33
		Total	59 Ave.	47	41	39	31
Immunized group							
γ -glob.b/P-3.F	1.5	1	15	80	80	80	60
" /P-3.N	1.5	1	15	100	93	93	67
" c/P-3.F	1.0	2	28	79	68	68	50
" b/P-3.F	1.5	3	17	100	100	100	100
" c/P-3.F	1.5	4	17	88	82	71	65
		Total	92 Ave.	87	83	80	66
Control* (spont. reg.)			31	100	100	90	68

* Control for spontaneous regression of S 37; no globulin or polysaccharide administered.

bearing mice receiving 50 γ of polysaccharide alone succumbed more readily than those which were given only 10 γ (see Table VII of the preceding paper).

To demonstrate that passive immunization did more than merely delay the lethal action of the polysaccharide, the per cent survival at 10 days has been listed also. It is evident that the majority of deaths due to the polysaccharide occurred within 24 hours. Although a few more mice died in the 24 to 96-hour period, there were not many deaths in the 4-10 day interval after the administration of the polysaccharide. A fairly high percentage of polysaccharide-treated mice died from the tenth to the 21st day. Almost the same percentage of deaths during the last two periods was noted also in the tumor-bearing mice serving as the spontaneous regression control group. In both cases, these delayed deaths were considered to be primarily the result of the growth of the tumor.

It is of interest to note that this protection was accomplished with γ -globulins (b) and (c) toward P-3.F. As stated before, these fractions were much lower in antibody content than γ -glob.a/P-3.F. as shown both by the serological evidence and by the

fully. Also, the extent of tumor regression at 21 days in the immunized and non-immunized mice receiving the polysaccharide was determined.

Dr. Diller's cytological examination of the tumors showed that the use of γ -globulin fractions of the rabbit antisera to decrease the lethal action of P-3.F did not prevent the characteristic cytological response of the tumors toward the action of the polysaccharide. Tumors taken from the passively immunized mice 6 hours after the polysaccharide injection were macroscopically hemorrhagic either peripherally or throughout the tumor. The maximum response occurred more slowly than it did in the control series treated with polysaccharide alone. At 6 hours, the tumor cells had reached a stage usually produced by the polysaccharide alone at 2 or 3 hours after treatment. Cell division persisted somewhat longer than usual, but the end result in the passively immunized mice was essentially the same as that produced by polysaccharide alone; hemorrhage and pronounced nuclear degeneration were produced in both groups.

Because of the high percentage (13 per cent) of spontaneous regression of the tumors in the untreated control mice, it is difficult to draw conclu-

sions about the extents of regression in the immunized and non-immunized mice treated with polysaccharide. Of the total of 100 mice which did not succumb to the lethal action of the polysaccharide within 4 days (Table III), approximately 50 per cent in both the immunized and non-immunized groups showed complete regression of their tumors when examined at autopsy 21 days after the polysaccharide had been administered. From the observations of the cytological responses of the tumors and from the trend of the regression rates, it would appear that the γ -globulin fraction of rabbit antisera toward the polysaccharide P-3.F had no detectable depressing influence on the tumor-necrotizing action of the P-3.F polysaccharide under the conditions of the experiment.

It has also been found that injection of antibody-containing fractions toward the P-10 polysaccharide into Swiss mice bearing sarcoma 37, prior to the injection of 250 γ of P-10, decreased the mortality rate to 22 per cent from that of 70 per cent ordinarily occurring when no passive immunization was employed. A complete report of the latter studies will be made when further work is done and after more nearly quantitative methods have been established for the estimation of cytological damage to the tumors.

Extensive work is in progress to establish the nature of the pronounced tolerance which is established within 24 hours after the initial injection of polysaccharide. This problem is particularly interesting because of the non-specific nature of this tolerance as developed in mice. It has been found that the injection into normal mice of a single non-lethal dose of 100 γ of the polysaccharide from either strain of organism afforded pronounced protection against the action of an ordinarily 90 per cent lethal dose of polysaccharide, either from the same or different strain, when administered 24 to 96 hours later. This was an unexpected finding in view of the specificity of action displayed by rabbit antisera toward the polysaccharides from the different strains of *Serratia marcescens*.

SUMMARY

Passive immunization of normal mice with the antibody-containing γ -globulin fraction isolated from rabbit antisera toward the P-3 type polysac-

charide from the G.W. strain of *Serratia marcescens* has been found to decrease the lethal activity of that polysaccharide but not that of the P-5 and P-10 polysaccharides from the 724 strain. The γ -globulin fractions of antisera toward the P-5 and P-10 polysaccharides from the 724 strain were either ineffective or only slightly effective in protecting normal mice against the lethal action of these polysaccharides. In Swiss mice bearing well-established sarcoma 37 transplants, it was demonstrated that passive immunization with antibody-containing fractions toward the G.W. strain polysaccharide, prior to the administration of relatively large doses of that polysaccharide, definitely decreased the mortality rate without interfering significantly with the tumor-necrotizing action of the polysaccharide.

ACKNOWLEDGMENTS

The authors wish to express their appreciation of the critical interest and encouragement of Dr. M. J. Shear and Dr. S. P. Reimann during the course of the investigations described in this and the preceding paper. Part of the experimentation described in this paper was supported by an American Cancer Society grant recommended by the Committee on Growth of the National Research Council.

REFERENCES

1. AVERY, O. T., and GOEBEL, W. F. Chemo-Immunological Studies on Conjugated Carbohydrate-Proteins. V. The Immunological Specificity of an Antigen Prepared by Combining the Capsular Polysaccharide of Type III Pneumococcus with Foreign Protein. *J. Exper. Med.*, **54**:437-447. 1931.
2. CREECH, H. J., HAMILTON, MARY ALICE, and DILLER, IRENE COREY. Comparative Studies of the Immunological, Toxic and Tumor-Necrotizing Properties of Polysaccharides from *Serratia marcescens* (*Bacillus prodigiosus*). *Cancer Research*, **8**:318-329. 1948.
3. LANDSTEINER, K. The Specificity of Serological Reactions. Cambridge, Mass.: Harvard University Press. 1945, p. 108.
4. ROSENTHAL, S. M. Experimental Chemotherapy of Burns and Shock. V. Therapy with Mouse Serum and Sodium Salts. *Public Health Reports*, **58**:1432-1436. 1943.
5. SHWARTZMAN, G. Reactivity of Malignant Neoplasms to Bacterial Filtrates. II. Relation of Mortality to Hemorrhagic Necrosis and Regression Elicited by Certain Bacterial Filtrates. *Arch. Path.*, **21**:509-523. 1936.

Carcinomas of the Uterine Cervix and Vagina in Estrogen- and Androgen-Treated Hybrid Mice*

S. C. Pan, M.B.** and W. U. Gardner, Ph.D.

(From the Department of Anatomy, Yale University School of Medicine, New Haven 11, Connecticut)

(Received for publication February 4, 1948)

Carcinomas of the uterine fundus occur more frequently among women showing unusual production of estrogenic hormone than in normal women (15). Although there is less evidence of a correlation of abnormal production of estrogens and tumors of the uterine cervix, at least one investigator has reported that deficiencies of vitamins, with possible coincident impairment of estrogen inactivation, was not unusual in women with precancerous lesions and early cancer of the cervix (2).

Invasive epithelial lesions (1, 6) 'precancerous' lesions (3, 10, 14) and carcinomas of the uterine cervix appear in estrogen-treated mice of all strains that have been adequately studied (5, 11, 12). Carcinomas of the cervix rarely occur in mice of most inbred strains, and none have been described that have arisen specifically from the cervix other than in mice from the PM stock, studied recently in this laboratory (9). Tumors of the genital tract of treated and control hybrid mice derived from crossing the PM stock¹ with mice of the C3H strain are reported here.

MATERIALS AND METHODS

Reciprocal hybrid mice were used; group PC₁ (C3H ♀ × PM ♂), and group PC₂ (PM ♀ × C3H ♂).¹ Fifty-seven female mice of the PC₁ group were used, 14 received estrogens and 43 were untreated controls. Ten of the 14 treated mice lived for more than 200 days and are included in this study (Chart I). Of the 70 female mice of the PC₂ group, 31 received steroid hormones and 39 were untreated. Twenty-five of the treated mice survived 200 days or more and are included in this study (Chart II).

* This investigation was aided by grants from The Anna Fuller Fund and the Jane Coffin Childs Memorial Fund for Medical Research.

** Anna Fuller Fund Fellow.

¹Mice of the PM stock were derived from a pair of mice received from Dr. H. B. Andervont and were descendants of mice that he had received from Drs. Pybus and Miller. The mice of the C3H strain were derived from animals received from Dr. L. C. Strong.

All mice were maintained in an air conditioned laboratory and fed Purina fox chow and water *ad libitum*. Prior to weaning, fresh lettuce was placed in their cages once or twice weekly. After weaning, the young were divided into hormone-treated and control groups. Four or 5 females and 1 or 2 male mice were quartered in each cage occupied by the controls; young born in the cage were removed at birth so that pregnancies were repeated frequently. The hormone-treated mice received either (a) 16.6 µgm. or 25 µgm. of estradiol benzoate, (b) 0.25 mgm. of stilbestrol, (c) 16.6 µgm. of estradiol benzoate plus 1 mgm. of testosterone propionate² weekly or one pellet weighing from 4.2 to 4.6 mgms. and composed of stilbestrol (one part) and cholesterol (3 parts). All injections were made subcutaneously and the pellets were implanted subcutaneously. Treatment was started when the mice were 25 to 65 days of age and continued until the animals died, had tumors or their general condition indicated the imminence of death. The period of treatment ranged from 174 to 594 days.

At necropsy all viscera and glands were examined and many tissues were preserved in Bouin's fluid or 10 per cent formalin. The uterine cervixes were usually sectioned serially and 2 of every 10 sections were mounted and stained in hematoxylin and triosin. The lesions were grouped in stages as described previously (1, 6).

OBSERVATIONS

Four of 10 mice of the PC₁ group that tolerated the treatment more than 200 days had invasive cervical lesions or carcinomas, whereas in the treated mice of the PC₂ group there were observed 9 carcinomas or invasive lesions, 8 arising from the uterine cervix and 1 from the vagina (Table I). All of the

²The estradiol benzoate and testosterone propionate used in this investigation were supplied by Dr. E. Schwenk of the Schering Corporation, and the stilbestrol was supplied by E. R. Squibb & Sons, New Brunswick, N. J. The stilbestrol-cholesterol pellets were made by a method similar to that described by T. R. Forbes (4).

TABLE I: CERVICAL CARCINOMAS OR INVASIVE LESIONS AMONG HYBRID MICE (PM × C3H) THAT RECEIVED SEX HORMONES

Group of mice	Mouse No.	Age at death, days	Level of treatment Weekly mgm.	Total mgm.	Period of treatment, days	Location	Stage†	Local invasion	Morphology Acute	Vascular lesions Chronic	Remarks
PC ₁	5	391	EB* 0.0166	0.830	356	Cervix	4	+		Hyaline change	Mammary tumor
	16	282	"	0.598	252	"	2	+	Endothelial proliferation		Lymphosarcoma and mammary cancer
	24	349	"	0.747	318	"	4	+	Necrosis and leukocytic infiltration	Endothelial prol. thrombosis	Hydronephrosis
	30	265	"	0.725	240	"	1	—			Lymphosarcoma
	11	396	"	0.830	354	"	1	—			
	12	417	"	0.879	375	"	1	—	Endothelial proliferation		Lymphosarcoma
	18	474	"	1.013	433	"	4	+	End. prol. § and necrosis		Lymphosarcoma
	42	310	EB .025	0.950	272	"	3	+	End. prol.		
	49	394	"	1.275	363	Vagina	3	—	End. prol. and occlusion		Hydronephrosis
	90	681	EB .0166 TP† 1.	1.544 93	650	Cervix	4	+	Fibrinoid degeneration and hemorrhage	End. prol. and occlusion	Adenoma of lung
	91	585	"	1.311 79	554	"	4	+		Hyaline change	
PC ₂	93	612	"	1.378 83	581	"	4	+		Hyaline and occlusion	
	96	658	"	1.411 85	592	"	1	—			

* EB = estradiol benzoate † TP = testosterone propionate ‡ See text for elaboration § Endothelial proliferation.

small lesions and apparently the carcinomas, except for one from the vagina, arose from the uterine cervical region with a predilection for the fornices of the vagina and posterior lip of the cervix. All lesions were composed of epidermoid cells that showed a lack of polarity, even in the small lesions, but that varied in the extent of invasion of adjacent tissue. Six among these 11 tumors were classed as stage 4 and considered to be carcinomas; they had extended beyond the cervix and vagina to adjacent tissues; 2 were grouped in stage 3 and had extended to the limits of the cervix; 1 in stage 2, and 2 in stage 1. Some of the carcinomas showed a cyst-like arrangement of the cells with cornified or mucoid material mixed with leukocytes in the cystic structure. Some of the tumors were composed of irregular sheets or solid strands of anaplastic or undifferentiated epithelial cells (Figs. 1, 2). Mitoses were frequently seen in these areas. In the larger tumors the entire uterine wall was invaded and the greater part of the genital tract was involved. Tumor cells invaded the lymphatics, blood vessel walls (Fig. 3) and the nerve sheaths (Fig. 4). The vaginal and cervical epithelia in the areas from which the smaller growths arose were thin, of low stratified epithelium showing no cornified layer. The epithelium of adjacent areas of the cervix and vagina was

thicker and usually slightly hyperplastic and often showed mucoid degeneration. Marked hyalinization of the basement membrane of the nontumorous epithelium was observed in one animal. Marked mucoid or hyaline change of the stroma, and occasional hyperplasia of the fibrous tissue were noted in many of the neoplastic or cancerous, as well as in non-neoplastic portions of the genital tract. In the majority of the animals the musculature of the uterine wall was replaced partially by scar or hyaline tissue.

Hyaline material also appeared along the walls of the lymphatics and blood vessels. In many instances both chronic and acute lesions of the parametrial and other arteries were encountered. In some areas the walls of the blood vessels were edematous; the endothelium was elevated and the media poorly defined; extreme proliferation of the intima, deposits of hyaline material or fibrinoid degeneration of the subendothelial tissue also occurred so that the lumina of some blood vessels were completely or partially occluded. Fibrous proliferation of the adventitial tissue was prominent and the cellular elements were infiltrated with mononuclear cells (Fig. 5). In one case hyalinized thrombotic material was mixed with the proliferating endothelial cells. Another artery in the same

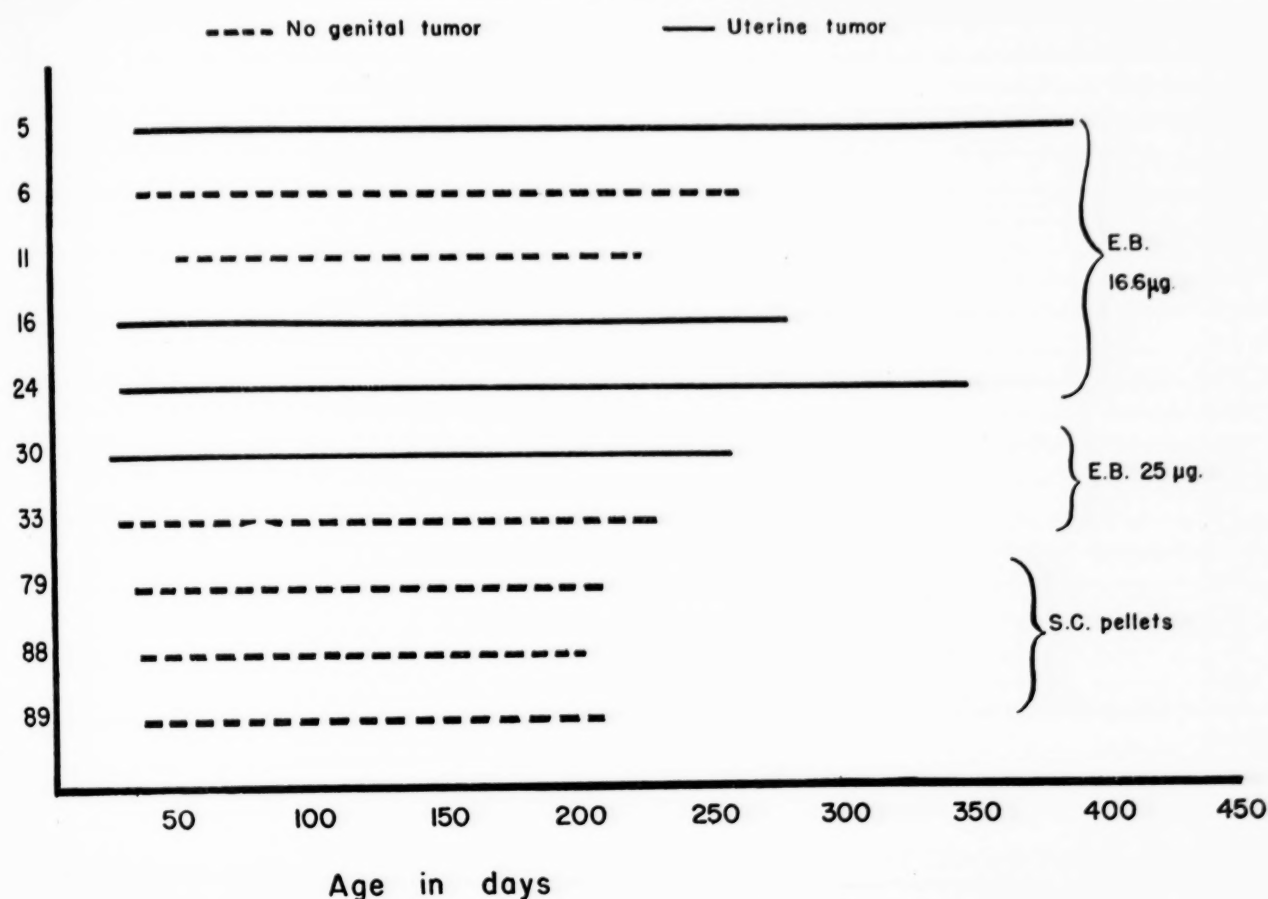


CHART I.—Age, treatment, and incidence of uterine cervical tumors in estrogen-treated hybrid mice ($C3H\eta \times$

PM δ). E.B. = estradiol benzoate. S.C. = pellets of stilbestrol, 1 part, and cholesterol, 3 parts.

region revealed necrosis and leukocytic infiltration of a part of its wall. Occasionally the walls of the blood vessels were impregnated with red blood cells.

Most tumors showed considerable infiltration of mononuclear leukocytes and plasma cells and occasionally ulceration had occurred.

Cystic hyperplasia of the endometrium of the uterine horns was noted in many animals, and in some, the cysts penetrated the musculature and were in contact with the serosa. The stroma of the uterine horns revealed mucoid change in some cases and the blood vessels had walls thickened by intimal and adventitial proliferation and medial degeneration. Similar vascular lesions and extreme narrowing or even occlusion of the blood vessels occurred in some of the ovaries.

The adrenal glands revealed nothing unusual. Two mice had associated hydronephrosis, 2 had mammary tumors and 1 had adenoma in the lung. Lymphosarcomas occurred frequently in mice of these groups; among 13 animals with cervical carcinomas, 4 had lymphosarcomas.

None of the 82 mice in the control groups had

cervical or uterine carcinoma, although 5 had uterine fibroids or fibromyomas; 1 in the PC_1 group at 508 days of age and 4 in PC_2 group at an average age of 462 days. Grossly the fibromyomas varied from 0.8 to 1.5 cm. in diameter and were encapsulated. In places they replaced the walls of the uterine horns (Figs. 6, 7). The tumors were composed of irregularly interlacing bundles of muscle and fibrous tissues within a fibrous capsule. In some areas marked hyaline change was noted. The mucosa overlying the tumor was thinner than usual and had a reduced number of glands. The endometrium of the remaining uterine horn revealed slight atrophy or occasional cystic hyperplasia. The lumina of the cornua were narrower than usual. The blood vessel walls were thick and hyalinized, and in one animal marked cellular proliferation of the endothelium with impregnation of red blood cells in the subendothelial region was observed.

DISCUSSION

Earlier experiments have shown that uterine cervical and vaginal tumors occurred in estrogen-

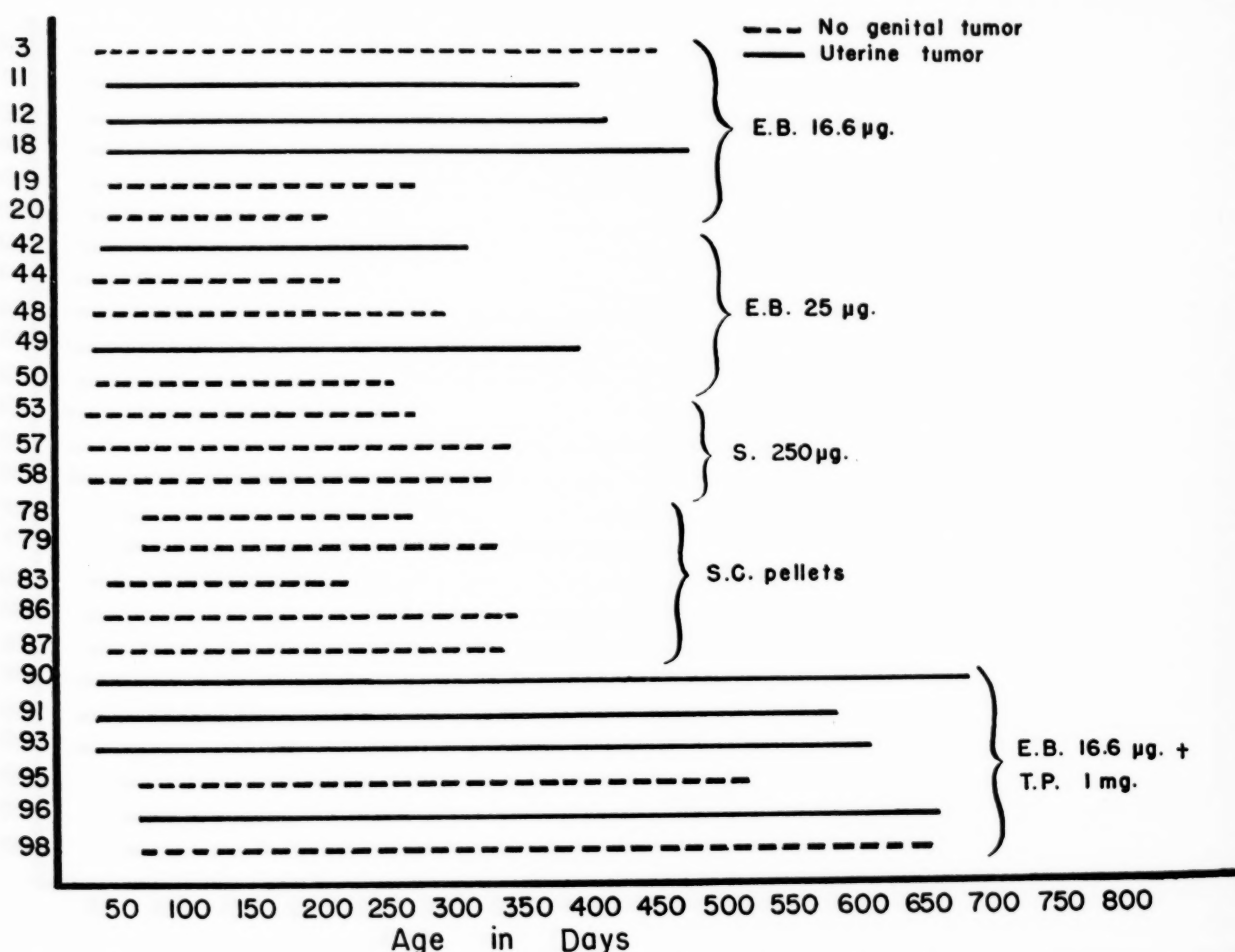


CHART II.—Age, treatment, and incidence of uterine cervical tumors among estrogen-treated hybrid mice (PM♀ × C3H). E.B. = estradiol benzoate. S = stilbestrol in oil.

S.C. = pellets of stilbestrol, 1 part; and cholesterol, 3 parts.
 T.P. = testosterone propionate.

treated mice and it has been reported that no hereditary tendency to develop carcinoma of the cervix had been observed. Among mice of the present groups the incidence of epidermoid carcinoma is higher than that in the untreated mice of the PM stock from which mice of these two groups descended. It is also slightly greater than that occurring in mice of other hybrid stocks and is comparable with that of the hybrid mice of CC₁ or CC₂ groups that survived treatment with estrogens for long periods (1). The duration of treatment was more important than the amount of the estrogenic chemicals within the range of dosage used. The average period of treatment preceding cervical neoplasia was shorter than in animals in other groups; no cervical lesions appeared in either CC₁ or CC₂ groups before 400 days of age. More than half of the tumors occurred within one year or less and one animal had a stage 3 lesion when it died at 272 days. Moreover about half of the tumors were

classed as carcinomas and considered to be malignant as indicated by invasiveness. The average period of survival of mice in the two groups was 46 and 108 days shorter respectively than that of the control animals, none of which had cervical or vaginal carcinomas or invasive lesions. Over 40 per cent of the estrogen-treated mice died with lymphoid tumors at relatively early ages (8).

Mice of the group treated with stilbestrol-cholesterol pellets did not have uterine cervical cancer, apparently because of their shorter life span (Charts 1 and 2). Mice given the larger doses of estradiol benzoate (25 µ weekly) acquired no higher percentage of cervical cancer than did those given smaller amounts. Half of the mice that received both estrogen and androgen and that survived more than 500 days had malignant tumors. Administration of the androgen reduced the incidence of lymphoid tumors, a possible cause of the increased longevity and the high incidence of cervical cancer.

As reported previously the untreated mice of the PM stock had a number of malignant tumors of undifferentiated cell type, probably carcinoma. This type of tumor was lacking in the estrogen-treated mice of the PC₁ and PC₂ groups, and the reason has not been determined.

In association with the neoplasia of the epithelium and the marked retrogressive lesions of the stroma, the chronic proliferative and obstructive vascular lesions in the uterine wall are of interest. Hyaline change and occlusion of the blood vessels have previously been observed in estrogen-treated mice in this laboratory (1, 6). Cellular proliferation and occlusion of the blood vessel walls were exceedingly prominent and involved the larger arteries in the parametrium and the vaginal and cervical walls. The so-called endarteritis obliterans type of lesions has been known to occur in various conditions and also in the genital tract during advanced age and is generally believed to be a result of disuse atrophy. Whether certain underlying hormonal imbalances or hereditary factors might have played a role and whether they were the causative or coincident findings in the tumorous animals is unknown.

The untreated mice of the PC groups did not have carcinomas of the genital tracts, in this respect being quite unlike the mice of the parental PM stock. The relatively high incidence of uterine fibromyomas was of interest because such tumors have not been noted so frequently in mice of other hybrid groups or strains in this laboratory. The appearance of fibromyomas in the old mice and their absence in the estrogen-treated mice, whereas cervical tumors did appear in the latter, indicates a dissimilar etiology of the two types of growth.

SUMMARY

Four of 10 estrogen-treated hybrid mice (C3H ♀ × PM ♂) that tolerated the treatment more than 200 days had carcinomas of the uterine cervix or vagina. Nine cervical carcinomas or invasive epithelial lesions were observed among 25 treated hybrids (PM ♀ × C3H ♂) that survived 200 days or more. All the 13 tumors were epidermoid carcinomas or invasive epithelial lesions that were considered to be early stages in tumor formation. None of the 82 mice in the control groups had carcinomas of the uterus or vagina although 5 had uterine fibromyomas. In association with the neoplastic change of the uterine and cervical tissue, chronic proliferative vascular lesions have been observed involving the parametrial and uterine vessels.

REFERENCES

1. ALLEN, E., and GARDNER, W. U. Cancer of the Cervix of the Uterus in Hybrid Mice Following Long-Continued Administration of Estrogen. *Cancer Research*, **1**:359-366. 1941.
2. AYRE, J. E., and BAULD, W. A. G. Thiamine Deficiency and High Estrogen Findings in Uterine Cancer and in Menorrhagia. *Science*, **103**:441-445. 1946.
3. CROSSEN, R. J., and LOEB, L. Effect of Long-Continued Administration of an Estrogen on the Sex Organs of Mice Which Have Passed the Reproductive Period. *Arch. Path.*, **37**:202-212. 1944.
4. FORBES, T. R. Absorption of Pellets of Crystalline Testosterone, Testosterone Propionate, Methyl Testosterone, Progesterone, Desoxycorticosterone and Stilbestrol Implanted in the Rat. *Endocrinology*, **29**:70-76. 1941.
5. GARDNER, W. U. Studies on Steroid Hormones in Experimental Carcinogenesis. *Recent Progress in Hormone Research*. Proc. Laurentian Conference, Vol. 1. New York: Academic Press, Inc. 1947, pp. 217-259.
6. GARDNER, W. U., and ALLEN, E. Malignant and Non-Malignant Uterine and Vaginal Lesions in Mice Receiving Estrogen and Estrogens and Androgens Simultaneously. *Yale J. Biol. & Med.*, **12**:213-234. 1939.
7. GARDNER, W. U., ALLEN, E., SMITH, G. M., and STRONG, L. C. Carcinoma of the Cervix of Mice Receiving Estrogen. *J.A.M.A.*, **110**: 1182-1183. 1938.
8. GARDNER, W. U., and DOUGHERTY, T. F. The Leukemogenic Action of Estrogen in Hybrid Mice. *Yale J. Biol. & Med.*, **17**:75-90. 1944.
9. GARDNER, W. U., and PAN, S. C. Malignant Tumors of Uterus and Vagina in Untreated Mice of the PM stock. *Cancer Research*, **8**:241-256. 1948.
10. LOEB, L., BURNS, E. L., SUNTZEFF, V., and MOSKOP, M. Carcinoma-like Proliferation in the Vagina, Cervix and Uterus of a Mouse Treated with Estrogenic Hormones. *Proc. Soc. Exper. Biol. & Med.*, **35**:320-322. 1936.
11. PERRY, I. H. Production of Carcinoma of the Uterus in Mice. *Proc. Soc. Exper. Biol. & Med.*, **35**:325-326. 1936.
12. PERRY, I. H., and GINZTON, L. L. The Development of Tumors in Female Mice Treated With 1:2:5:6-Dibenzanthracene and Theelin. *Am. J. Cancer*, **29**:680-704. 1937.
13. STRONG, L. C. The Origin of Some Inbred Mice. *Cancer Research*, **2**:531-539. 1942.
14. SUNTZEFF, V., BURNS, E. L., MOSKOP, M., and LOEB, L. On the Proliferative Changes Taking Place in the Epithelium of Vagina and Cervix of Mice with Advanced Age and Under the Influence of Experimentally Administered Estrogenic Hormones. *Am. J. Cancer*, **32**:256-289. 1938.
15. TAYLOR, H. C. Endocrine Factors in the Origin of Tumors of the Uterus. Chap. V. *Endocrinology of Neoplastic Diseases*. New York: Oxford University Press. 1947, pp. 119-137.

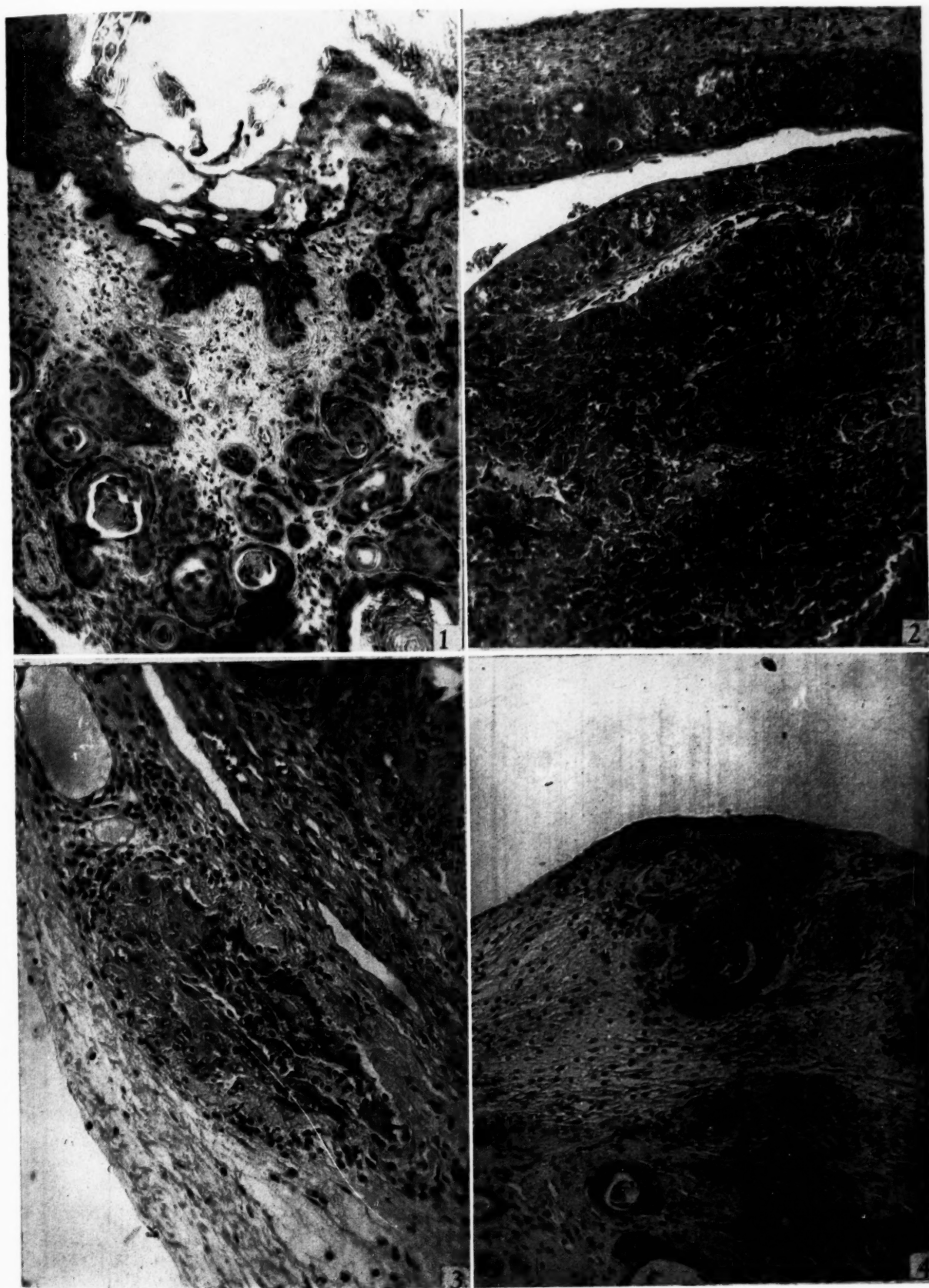
DESCRIPTION OF FIGURES 1 TO 4

FIG. 1.—Photomicrograph showing junction of normal cervical epithelium (upper left) with the epithelium over the infiltrative lesion in 24PC₁.

FIG. 2.—A section through the vaginal fornix of a mouse (91PC₂; Table I). The irregular basement membrane and folded arrangement of the non-tumorous epithelium (upper part of photograph) is common in mice given testosterone propionate and estrogen. An anaplastic tumor is shown in the lower part of the photograph.

FIG. 3.—An extension of tumor cells perivascularly into the outer vaginal wall (Stage 3 tumor; 49PC₂; Table I).

FIG. 4.—Extension of the tumor beyond the cervical wall and proliferative and degenerative lesions of adjacent arteries (18PC₂; Table I).



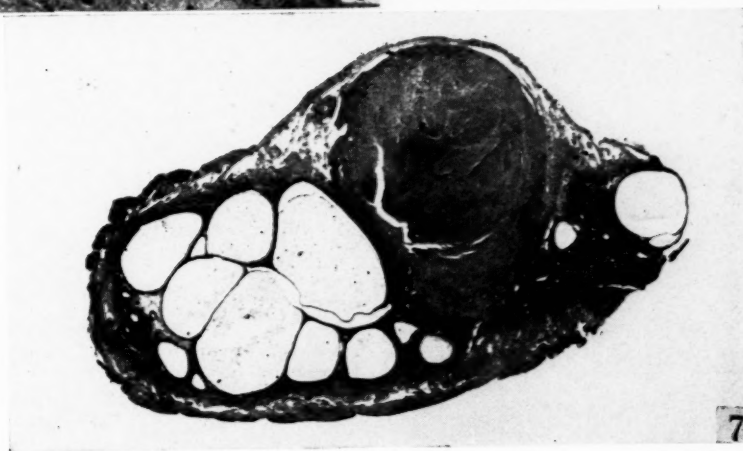
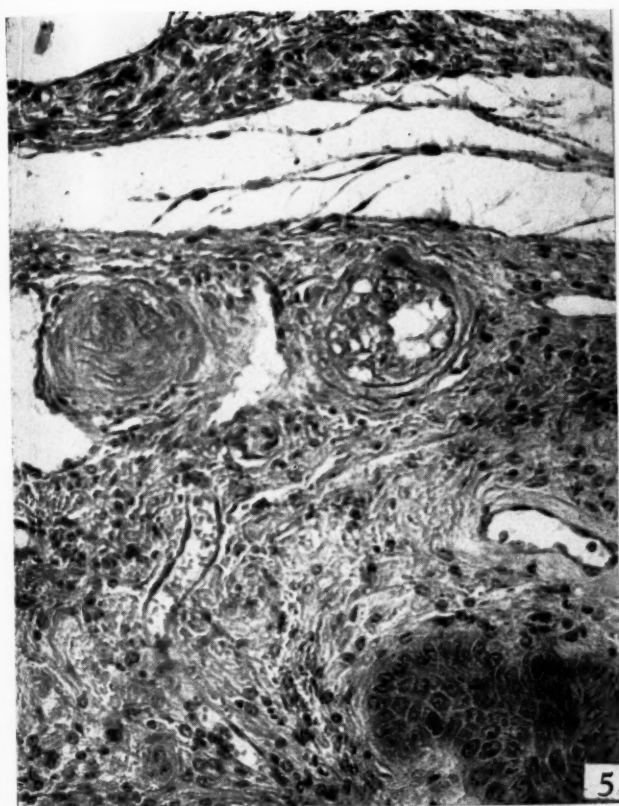
FIGS. 1-4

DESCRIPTION OF FIGURES 5 TO 7.

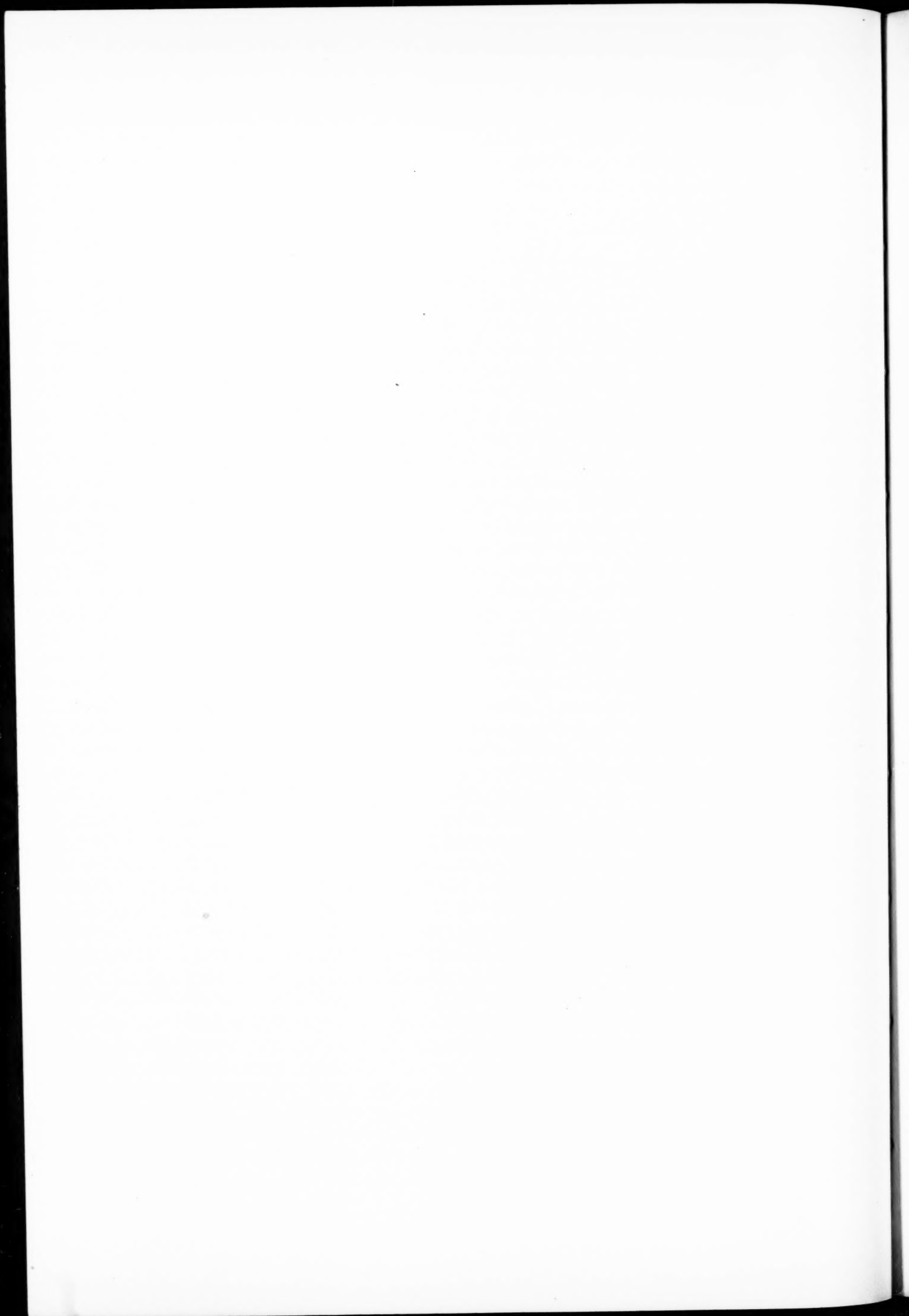
FIG. 5.—Obliterative arterial lesion in the parametria as a result of intimal proliferation. (One artery reveals marked intimal proliferation with narrowed lumen and the other artery has a completely obliterated lumen.)

FIG. 6.—Fibromyoma of the uterine horn of a mouse (46 PC.) in the control group. The tumor is well encapsulated. The endometrium shows cystic glands.

FIG. 7.—Photomicrograph of the same tumor revealing the interlacing bundles of fibrous tissue and the overlying endometrium.



FIGS. 5-7



The Effect of Methylcholanthrene on the Viscosity of Paramecium*

Joseph M. Butros, M. A.

(From the Department of Histology, School of Medicine, American University of Beirut, Beirut, Lebanon)

(Received for publication November 24, 1947)

Following the method of Heilbrunn (5, 6), Fetter (3) determined the viscosity of *Paramecium* by feeding particles, such as starch, to paramecia and centrifuging them until the particles accumulate at one end of the organism. Fetter calculated the absolute viscosity of paramecium from Stokes' law which gives the viscosity when the centrifugal force, the size, speed, and specific gravity of the particles are known. For determinations of relative viscosities, it is sufficient to centrifuge and note the time needed to concentrate ingested particles within the animal. By such methods Angerer (1) found that slowly applied thermal increments produced a slow decrease in viscosity; whereas sudden increments within the same range produced a sharp decrease in the viscosity.

King and Beams (7) found that mechanical agitation causes a decrease in the viscosity of paramecium. The paramecia were fed powdered carmine after which they were agitated by shaking. On centrifuging they found that the carmine particles were packed in one end of the agitated paramecia in less time than in the controls.

Guyer and Claus (4) using the centrifuge method determined the relative displacement of mammalian cellular constituents in tumor cells as compared with normal cells. They concluded that the viscosity of the tumor cells was higher than that of the normal cells; however, Cowdry and Paletta (2) found a decrease in the viscosity of nuclei of cancerous cells of mouse epidermis.

Carcinogenic agents such as methylcholanthrene have also been tried on paramecium and morphological changes and aberrations in mitosis have been described (8-10).

It is the purpose of this paper to describe the effect of methylcholanthrene on the viscosity of the endoplasm of paramecium.

EXPERIMENTAL PROCEDURE

The pure line of paramecium used in this work was cultured in hay infusion and subcultured every 10 days. The culture was divided into three groups:

*This investigation was aided by a grant from the Cancer Research Division of the Donner Foundation.

to the first, 15 mgm. of methylcholanthrene (Eastman Kodak Co.) was added; to the second, the same amount of the noncarcinogenic anthracene; and the third was left as a hay infusion control. The compounds were given as finely powdered suspension or were first dissolved in a few drops of benzene. After evaporation of the solvent, they were emulsified in hot water and added to the culture. After the paramecia had been growing for at least a month in the three media, experiments were made to determine differences in viscosity. The method consisted of feeding the paramecia corn starch for a half hour, after which a sample from each of the three cultures was taken and centrifuged at various speeds. The animals were then killed with iodine. The time required for the concentration of the starch particles at one end of the animal was compared in the three cultures, and this time was taken as a measure of the relative internal viscosity.

The pH of the culture media was measured at different times, but no significant differences were found between the three cultures. For example, the pH's of the media during one of the experiments were as follows: methylcholanthrene culture, 7.60; anthracene culture, 7.60; hay infusion culture 7.56.

During these experiments heat, direct sun rays, agitation, and other factors that might influence viscosity were avoided. In order to avoid the possibility that no changes in viscosity were momentary or periodic, data were collected at various age periods.

RESULTS

There were two series of experiments, each with a different centrifugal speed.

A. In the first set of experiments a speed of 2,000 r.p.m. was employed. The average time, based on the examination of at least 100 paramecia in each experiment, necessary to concentrate the starch particles at one end of the animal varied between 40 and 50 minutes of centrifuging (Table I). After 40 minutes 75 per cent of the methylcholanthrene-treated organisms displayed concentrated masses, whereas no similar masses were observed in the 2 controls. After 50 minutes almost

all the individuals from the methylcholanthrene culture showed a concentrated mass of starch particles in contrast to 50 per cent for the hay infusion and anthracene control cultures.

B. In the second group of experiments, in order to shorten the time interval for the concentration of the particles, the paramecia in this second set were

TABLE I: FIRST SET OF EXPERIMENTS. RELATIVE RATE OF CONCENTRATION OF STARCH PARTICLES IN PARAMECIA CENTRIFUGED AT 2,000 R.P.M.

Time, Min.	Methylcholanthrene culture	Anthracene culture	Hay infusion culture
15	No concentration	No concentration	No concentration
20	No concentration	No concentration	No concentration
30	Concentrated masses in 40%	No concentration	No concentration
40	Concentrated masses in 75%	No concentration	No concentration
50	Nearly all have concentrated masses	Concentrated masses in approx. 50%	Concentrated masses in about 50%

centrifuged at 4,000 r.p.m. Here also the number of paramecia examined from each sample was at least 100 in each case. The results (Table II) are based on many repeated experiments on the three cultures at different ages following the addition of the methylcholanthrene and anthracene; however, they remained at least a month in these compounds before the studies were made. The onset of the change in viscosity after the addition of the carcinogen was not studied. We found that when centrifuged for 7.5 minutes, at the higher speed, only the methylcholanthrene-treated organisms had starch particles concentrated at their ends. After 15 minutes' centrifuging both the methylcholanthrene-treated and the control animals exhibited particle-concentration at their ends. Thirty minutes of centrifuging produced concentration of the particles in one end, both in the treated organisms and the controls; however, many of the treated paramecia had a swollen vacuole bulging to the exterior.

TABLE II: SECOND SET OF EXPERIMENTS. RELATIVE RATE OF CONCENTRATION OF STARCH PARTICLES IN PARAMECIA CENTRIFUGED AT 4,000 R.P.M.

Time, Min.	Methylcholanthrene culture	Anthracene culture	Hay infusion culture
7.5	Concentration in all	No concentration	No concentration
15	Concentration in all	Concentration in all	Concentration in all
30	Bulging concentrated masses in all	Concentrated in all, but no bulging	Concentration, but no bulging

SUMMARY

1. Paramecia from a single line were cultured in hay infusion. Some of these were treated with the carcinogen, methylcholanthrene, and others with the noncarcinogenic anthracene.

2. Studies on relative viscosity of the endoplasms of the organisms were made by feeding them corn starch and noting the time required to accumulate the ingested particles into a compact mass at one end by centrifugal force.

3. In methylcholanthrene-treated paramecia, the accumulation into a compact mass was more rapid than in those from the hay infusion, or those treated with anthracene. This was interpreted as indicating a decrease in the viscosity of the methylcholanthrene-treated animals. Anthracene caused no change in the viscosity of the paramecia.

ACKNOWLEDGMENT

It is a pleasure to acknowledge the writer's indebtedness to Dr. W. M. Shanklin, Professor of Histology at the American University of Beirut, for his interest and guidance in this work.

REFERENCES

1. ANGERER, C. A. The Effect of Thermal Increments and the Subsequent Adjustment of the Protoplasmic Viscosity of *Amoeba Proteus*. *Physiol. Zool.*, **13**:219-226. 1940.
2. COWDRY, E. V., and PALETTA, F. X. Alterations in Nuclear Viscosity during Experimental Carcinogenesis Determined by Ultracentrifugation. *Am. J. Path.*, **17**:335-357. 1941.
3. FETTER, DOROTHY. Determination of the Protoplasmic Viscosity of Paramecium by the Centrifuge Method. *J. Exper. Zool.*, **44**:279-283. 1926.
4. GUYER, M. F., and CLAUS, P. E. Relative Viscosities of Tumor Cells as Determined by the Ultracentrifuge. *Anat. Rec.*, **73**:17-27. 1939.
5. HEILBRUNN, L. V. Protoplasmic Viscosity Changes during Mitosis. *J. Exper. Zool.*, **34**:417-447. 1921.
6. HEILBRUNN, L. V. The Absolute Viscosity of Protoplasm. *J. Exper. Zool.*, **44**:255-283. 1926.
7. KING, R. L., and BEAMS, H. W. Some Effects of Mechanical Agitation on *Paramecium Caudatum*. *J. Morphol.*, **68**:149-159. 1941.
8. MOTTRAM, J. C. Abnormal Paramecia Produced by Blastogenic Agents and Their Bearing on the Cancer Problem. *Cancer Research*, **1**:313-323. 1941.
9. SPENCER, R. R., and MELROY, M. B. Effect of Carcinogens on Small Organisms. III. Cell Division Rate and Population Levels of Methylcholanthrene-Adapted Paramecia. *J. Nat. Cancer Inst.*, **2**:185-191. 1942.
10. TITTLER, I. A., and KOBIRIN, MILDRED. Effects of Carcinogenic Agents on *Paramecium caudatum*. *Proc. Soc. Exper. Biol. & Med.*, **50**:95-96. 1942.